A Combined Chemical and Biological Approach to the Measurement of Chromium Speciation, Availability and Ecotoxicity in Contaminated Soils

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Abstract

With the increasing need for the re-development of contaminated urban areas, the establishment of rapid and reliable analytical tools to support risk assessment and remediation, has become an important issue in recent years. In order to account for the different factors contributing to the bioavailability and ecotoxicity of contaminants, a complete ecotoxicity screening should use a combination of chemical and biological analyses. Chemical analyses cannot by themselves provide ecotoxicological information, while microorganisms, despite having proved to be good indicators of the toxicity of aquatic and terrestrial systems, usually cannot describe their composition.

In this work the (eco)toxicity of chromium (Cr) in soil contaminated with Cr salts or chromite ore processing residue (COPR) was studied using chemical analysis and bioassays. Acute and chronic bioassays were used to compare the toxicity of Cr(VI) under short and long term exposure conditions. Bioassays used included: luminescent biosensor *E. coli* pUCD607, plant growth and root development, analysis of soil community structure by Signature Lipid Biomarkers (phospholipid fatty acid extraction and analysis) and analysis of soil metabolic capacities through Community Level Physiological Profiles.

It was found that acute and chronic bioassays gave different pieces of information, which in conjunction with chemical analysis could be more useful to determine the bioavailability and (eco)toxicity of Cr-contaminated soils, as well as possible contributory factors. The risks associated with contaminated sites could be better assessed by the use of this combined approach, which may also be useful for other type of contaminant.

...and because there was nowhere to go but everywhere,

keep rolling under the stars...

Jack Kerouac, *On the Road*, 1957.

Se nos acabo el veinte, aparentemente, pero como siempre les dedico esta quimera. Gracias por llevarme de la mano. A:

Clemencia Manzur Aguilar
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Introduction

1.1 An overview of chromium in the environment

1.1.1 Chromium history and uses

Chromium (Cr) is a transition element located in the group VI-B of the periodic table (element 24), with an average atomic weight of 52. At around 100 mg/kg, it is considered the 21st most abundant element in the Earth's crust (Barnhart, 1997).

Despite being more abundant than other well-known metals such as Cd and Zn, Cr was unknown to ancient civilisations. The history of Cr began with the discovery of the minerals crocoite ($PbO \cdot CrO_3$) and chromite $(Mg,Fe)(Cr,Al,Fe)_2O_4$ at the end of the 18th century in the Ural Mountains. The discovery of chromium in an ore from Siberia in 1797 is attributed to the French chemist Louis Vaquelin, (Marvin, 1956). He noticed that, whatever reagents he used in his experiments, the filtrates and precipitates were brilliantly coloured, green, yellow or red. For this reason he named the new metallic element chrome (chromium), from the Greek word *chroma* (colour).

For several years the only ores available were those from Siberia. The ore was difficult to transport, taking up to a year to arrive in central

Europe. It was not until the discovery of chromite ores near Baltimore, in the USA, and years later of ores in other regions, that the industrial mining of chromite began (Barnhart, 1997).

Chromite was first employed to make chromium chemicals. In England, Andreas Kurtz, a pupil of Vaquelin, started the manufacture of chromium chemicals in London in 1816. By 1820, John and James White in Glasgow, Scotland, started manufacturing potassium dichromate and began to specialise in other chrome materials. The White chemical works became one of the largest chromium chemical manufacturing plants worldwide (Farquhar, 1999).

Chromite was employed as a refractory for the first time in France in about 1879. The intensified use of chromium metal in steel started about 1908 to 1912. Chemical, refractory and metallurgical applications constitute the main uses of chromite ore today (Darrie, 2001).

While there are some direct uses of chromite ore, mainly for the production of refractory brick, the vast majority of ore is either oxidised or reduced.

The oxidation of chromite results in Cr(VI) compounds. Traditionally, sodium chromate (Na_2CrO_4) was produced by the air roasting (oxidation) of chromite with sodium carbonate and calcium oxide (lime), also yielding water-insoluble calcium chromate ($CaCrO_4$) as a by-product. Newer processes use low-lime or no-lime technologies (Langard, 1990). Dichromate ($Na_2Cr_2O_7$), chromic oxide (CrO_3), chromic acid ($H_2Cr_2O_7$) and other oxides of chromium, *e.g.* K_2CrO_4, including the chromate pigments (barium, calcium, lead, strontium

and zinc chromate) are in turn derived from the dichromate (Kimbrough *et al.*, 1999).

The first development in the use of chromium was in the chemical field. From the manufacture of potassium chromate and chromium green for paint pigments and ceramic colours, the uses of chromium chemicals soon widened into tanning, textiles colouring, corrosion prevention, chromium plating, graphic arts, fungicidal applications, organic synthesis, inorganic oxidations, electrochemical oxidations, and others (Darrin, 1956).

Chromite ore can also be reduced by a variety of methods using diverse reducing agents. The reduced materials can be used for the production of chromium alloys. Most of the chromium consumed worldwide is used for the production of metal alloys, mainly wrought-stainless and heat resistant steels. On a worldwide basis, about 80% of the chromium extracted from the ore is used in metallurgical applications (Barnhart, 1997).

1.1.2 Geochemistry of chromium

Chromium can exist in every oxidation state from –2 to +6, with the trivalent and the hexavalent forms being the most stable under environmental conditions (Shupak, 1991). The trivalent state is the most stable form under reduced conditions. Cr(III) is found naturally in several minerals, including chromite, copiapite, daubreelite, dietseite, fornacite, halotrichite, kaemmererite, lopezite, merumite, muscovite, phoeniocochroite, stichtite, barbetonite, uvarovite, vauquelinite, beidellite (Thayer, 1956). Some of these minerals are also found as soil components.

The presence of Cr(VI) in the environment is generally due to anthropogenic inputs, as only rarely has Cr(VI) been found in nature, and then in places with very unusual local environments (Barnhart, 1997). Chromium(VI) mineral phases such as $PbCrO_4$ (crocoite), $PbCrO_4.H_2O$ (iranite), K_2CrO_4 (tarapacaite), $CaCrO_4$, $BaCrO_4$ (hashemite) have been found in contaminated sites (Palmer and Puls, 1994).

1.1.2.1 Dissolution and precipitation reactions

Chromium can undergo precipitation-dissolution reactions. These reactions are governed by the solubility of the chromium compounds and the kinetics of the dissolution. The water solubility of both chromium (III) and chromium (VI) species varies over many orders of magnitude (Kimbrough *et al.*, 1999).

Chromium(III)

In the Cr(III)-H_2O system, Cr(III) exists predominantly as Cr^{3+} below pH 3.5. When the pH of the solution increases, the hydrolysis of Cr^{3+} takes place and the species $CrOH^{2+}$, $Cr(OH)_2^+$, $Cr(OH)_3^\circ$ and $Cr(OH)_4^-$ are formed (Rai *et al.*; 1987). Cr(III) can precipitate as an amorphous chromium hydroxide when slightly acidic to alkaline conditions exist. Amorphous $Cr(OH)_3$ may crystallise as $Cr(OH)_3.3H_2O$ or Cr_2O_3 (Swayambunathan *et al.*, 1989).

Chromium (III) can also precipitate as a solid solution in the presence of Fe(III). If the pH of the system is between 5 and 12, the aqueous concentration of Cr(III) should be less than 0.05 mg/L. The low solubility of Cr(III) at alkaline pH, coupled with its strong

retention on soil surfaces, limits its bioavailability and mobility in soils and waters (Fendorf, 1995).

Chromium(VI)

In solution, the anionic Cr(VI) species exist as monomeric ions $H_2CrO_4°$, $HCrO_4^-$ (hydrogen chromate) and CrO_4^{2-} (chromate), or as the dimeric ion $Cr_2O_7^{2-}$ (dichromate) (Richard and Bourg, 1991), depending on the pH of the solution and the total concentration of Cr(VI).

The monomeric chromate species are related through a series of acid dissociation reactions:

$H_2CrO_4° \leftrightarrows HCrO_4^- + H^+ \quad pK_1 = -0.86$
$HCrO_4^- \leftrightarrows CrO_4^{2-} + H^+ \quad pK_2 = 6.51$

The dichromate is the result of the polymerisation of the monomeric hydrogen chromate ions to form the dimer:

$HCrO_4- + HCrO_4^- \leftrightarrows Cr_2O_7^{2-} + H_2O \quad pK_d = -1.54$

The relative concentration of each of these species in solution depends on both the pH and the concentration of Cr(VI). The speciation diagram in Figure 1.1 shows that below pH 6.5, the dichromate and hydrogen chromate species dominate (the hydrogen chromate species is more abundant below 30mM Cr(VI); the dichromate species becomes significant when Cr(VI)>1 mM and may even dominate when Cr(VI)>30 mM). Above pH 6.5, CrO_4^{2-} generally dominates.

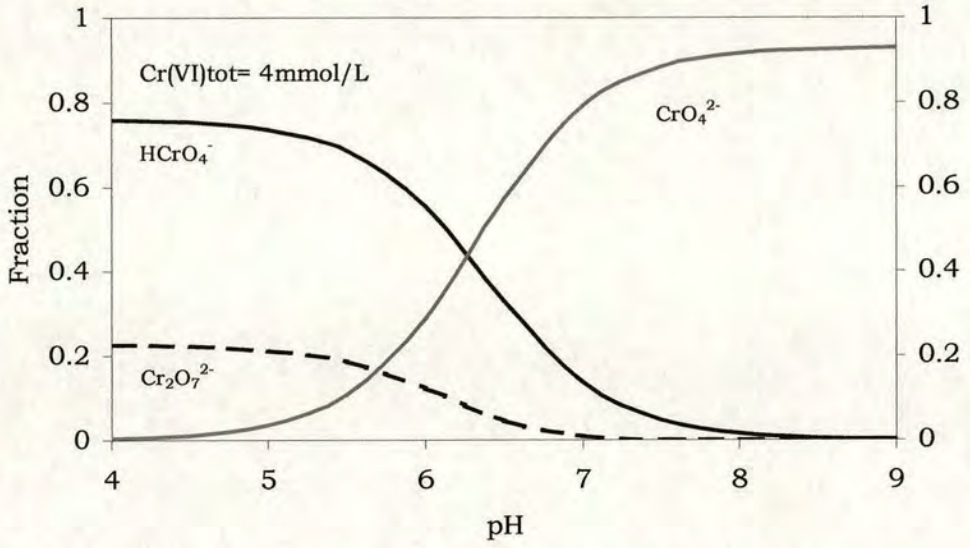

Fig. 1.1 Speciation diagram of Cr(VI) as a function of pH. Only major species represented.

In spite of chromate and dichromate ions being water-soluble at all pHs, insoluble salts of chromate can be formed with a variety of divalent cations (e.g. Ba^{2+}, Ca^{2+}, Sr^{2+}, Pb^{2+}, Zn^{2+} and Cu^{2+}) and these salts have a wide range of solubilities. The rates of precipitation/dissolution reactions between chromate and dichromate anions and these cations vary greatly and are pH dependent. An understanding of the dissolution reactions is particularly important for assessing the environmental effects of chromium because Cr(VI) often enters the environment by dissolution of chromate salts. Dissolution of sparingly soluble chromate salts, *e.g.* $SrCrO_4$, is important because they provide a continuous source of chromate anions (Kimbrough *et al.*, 1999).

1.1.2.2 Oxidation and reduction reactions

Chromium (III) and Cr(VI) can interconvert depending on several factors, including the presence and concentrations of the chromium species and oxidising or reducing agents, the electrochemical

potentials of the oxidation and reduction reactions, ambient temperature, light, sorbents, acid-base reactions, complexing agents and precipitation reactions (Saleh *et al.*, 1989). It has also been observed that certain microorganisms are capable of reducing Cr(VI) to Cr(III), either under aerobic or anaerobic conditions, although the mechanism is still not clear (Palmer and Puls, 1994).

Chromium(VI) is a strong oxidant and can be reduced by electron donors commonly found in soils (Kožuh *et al.*, 2000), including aqueous Fe(II) (Sedlak and Chan, 1997), ferrous iron minerals (Eary and Rai, 1989), sulfides (Kim *et al.*, 2001), and soil organic matter (Bartlett and Kimble, 1976; Kožuh *et al.*, 2000).

Even in the presence of dissolved oxygen, the reduction of Cr(VI) by ferrous iron is very fast on the time scales of interest for most environmental problems, with the reaction going to completion in less than 5 minutes (Eary and Rai, 1988). The reaction can be written as:

$$HCrO_4^{2-} + 3Fe^{2+} + 7H^+ \rightarrow Cr^{3+} + 3Fe^{3+} + 4H_2O$$

When the pH of the solution is greater than 4, Cr(III) precipitates with Fe(III) in a solid solution with the general composition $Cr_xFe_{1-x}(OH)_3$ (Palmer and Puls, 1994). Only when the pH of the solution is greater than 10 or when the concentration of phosphate exceeds 0.1 M, does the rate of oxidation of Fe^{2+} by dissolved oxygen exceed the rate of oxidation by Cr(VI).

Several minerals contain ferrous iron that is potentially available for the reduction of hexavalent Cr. These iron-containing minerals may be silicates (including olivine), pyroxenes (such as augite and hedenbergite), amphiboles (such as hornblende, cummingtonite and

grunerite), micas (such as biotite, phlogopite and glauconite), chlorites, and the smectite nontronite. In sulfide minerals such as pyrite (FeS_2), both the ferrous iron and the sulfide are active in reducing hexavalent chromium (Palmer and Puls, 1994). Chromium (VI) reduction in the presence of iron oxides and silicates has been observed experimentally (Eary and Rai, 1989).

Organic matter contains another important group of reducing agents for Cr(VI) in soils. Much of the soil organic matter is present as humic and fulvic acids. The reduction of Cr(VI) by soil humic and fulvic acids has been demonstrated by several authors (Bartlett and Kimble, 1976; Bloomfield and Pruden, 1980).

Dichromate can react with soil organic carbon according to:

$$2\ Cr_2O_7^{2-} + 3C + 16H^+ \rightarrow 4Cr^{3+} + 3CO_2 + 8H_2O$$

The resultant Cr^{3+} ion may hydrolyse and precipitate as Cr-hydroxide or it may bind with the remaining soil organic carbon.

The rate of reduction of Cr(VI) decreases with increasing pH and increases with the increase of Cr(VI) concentration and organic matter content. At neutral pH, many weeks may be required for Cr(VI) to be completely reduced to Cr(III).

There are very few mechanisms for the oxidation of Cr(III) to Cr(VI). Only two constituents in the environment are known to oxidise Cr(III) to Cr(VI): dissolved oxygen and manganese dioxide (MnO_2), but only the oxidation by MnO_2 seems to be of environmental importance and it has been experimentally verified using mineral phases (Eary and Rai, 1987). As the pH decreases, the rate and

extent of Cr(III) oxidation increases. Experimental results suggest that the oxidation follows the reaction:

$$CrOH^{2+} + 1.5\ \delta\text{-}MnO_2 \rightarrow HCrO_4^- + 1.5\ Mn^{2+}$$

The oxidation of Cr(III) has been observed in several soils (Bartlett and James, 1979; James and Bartlett, 1983; Palmer and Puls, 1994), but it can be relatively slow, requiring several months for completion.

1.1.2.3 Sorption and desorption reactions

Sorption and desorption reactions are important to understand the fate and transport of chromium in the environment. Chromium ions are attracted to surfaces that have a net electric charge due to imperfections or substitutions in the crystal lattice or due to chemical dissociation reactions at the surface of the solid.

Due to its anionic nature, Cr(VI) is not retained appreciably on negatively charged colloids of soils or sediments. Hydrous oxides of Al and Fe are often present at significant levels in surface environments; they commonly have a net positive charge and a potential chemical affinity for Cr(VI). Unlike for PO_4^{3-}, the retention mechanism of Cr(VI) is still not understood clearly (Fendorf, 1995).

Chromium (III) can bind to solids that have exposed, negatively charged silicates, while organic materials will sorb organically bound chromium.

As with redox and precipitation reactions, sorption reactions are highly influenced by the complex environmental conditions in a given medium; thus, generalised assumptions about sorption cannot

be made. Variables that can influence sorption equilibria are, amongst others, pH, surface area and density of active sites.

1.1.3 Entry points, transport and fate of chromium in the environment

Chromium is emitted into air, water systems or soils mainly as the result of industrial activity. Nriagu and Pacyna (1988) used emission factors to calculate the inputs of trace elements into the environment. They calculated that the most significant amounts of Cr were emitted into soils, followed by water and air (Table 1.1).

Airborne Cr eventually settles out into the soil or water bodies. Chromium (VI) can be leached out of the soil and contaminate adjacent land or enter the groundwater, which in turn can become part of an aquifer and also migrate to surface waters. As Cr(VI) is leached out from the soil, the remaining Cr(III) can slowly oxidise to Cr(VI) to re-establish the equilibrium of the soil (Bartlett, 1991).

In aqueous environments Cr(VI) can migrate in the dissolved form, while both Cr(III) and Cr(VI) can migrate bound to dissolved organic carbon (DOC) or suspended particles. In sediments, dissolved Cr(VI) can be immobilised if it enters a stable anoxic zone, but Cr(VI) in oxic sediments can be re-dissolved.

The Cr cycle in the environment begins and ends with its least mobile form, Cr(III), either precipitated or bound by a variety of ligands, such as hydroxyls, humates and phosphates, or in more inert forms, substituting for Fe or Al in mineral structures (Bartlett and James, 1993). In its cycling through the environment, chromium undergoes the series of reactions previously discussed.

Table 1.1 Major emissions of Cr into air, water and soils. Adapted from Nriagu and Pacyna, (1988).

Emissions into soils (10^6 kg/year)		Emissions into aquatic systems (10^6 kg/year)		Emissions to the atmosphere (10^6 kg/year)	
Agriculture and food wastes	4.5 – 90	Domestic wastewater	14.1-78	Coal combustion	2.9-19.6
Animal wastes and manure	10-60	Chemicals manufacturing	2.5-24	Oil combustion	0.4-2.3
Coal fly ash and bottom fly ash	149-446	Metal manufacturing	15-58	Steel and iron manufacturing	2.8-28.4
Wastage of commercial products	305-610	Atmospheric fallout	2.2-16	Cement production	0.8-1.7
Total input	484-1309	Total input	45-239	Total input	7.3-53.6

There is much evidence to show that chromium can be taken up by biota from the air, water and soil. Unfortunately most of the available studies on chromium bioaccumulation and bioconcentration report total chromium measured in the exposed organism without distinguishing between its oxidation states. Thus understanding of the biologically available fractions, bioconcentration factors and transfer coefficients for Cr(VI) are lacking (Kimbrough *et al.*, 1999).

1.1.4 Chromium transport and accumulation in microorganisms

Microorganisms have the potential to accumulate chromium (Coleman, 1988) and to reduce Cr(VI) to Cr(III) (DeLeo and Elrich, 1994). The transport of the chromate through sulphate transport system has been demonstrated in several microorganisms, including *Pseudomonas fluorescens* (Ohtake *et al.*, 1987) and *Alcaligenes eutrophus* (Nies *et al.*, 1989). Despite the fact that high levels of Cr(VI) are toxic to microorganisms (Bartlett, 1991), chromium is important to yeast metabolism, and sorption of Cr by several species of yeast has been reported (Rapoport and Muter, 1995), where Cr(VI) may enter the cells via a non-specific anion carrier, the permease

system, which transports different anions such as sulphate and phosphate (Borst-Pauwels, 1981).

1.1.5 Chromium transport and accumulation in plants

There are conflicting views concerning the uptake and translocation of Cr(VI) in plants, as the measurement of Cr species content has been difficult. Some authors have suggested that, as in microorganisms, the sulphate transport system is involved in CrO_4^{2-} uptake by plants, since sulphate competitively inhibits CrO_4^{2-} uptake in barley seedlings (Shewry and Peterson, 1974). Ramachadran *et al.* (1980) suggested that CrO_4^{2-} is reduced to Cr(III) at the surface of root cells. Other studies found Cr(VI) in plants, which suggests that dissolved chromium may be taken up by plants without immediate reduction (Misha *et al.*, 1995). However, Cary (1982) reports there is no evidence that Cr(VI) is translocated in the plants. Pulford *et al.* (2001) reported that the uptake of Cr in a range of tree species occurred mainly in the roots and was poorly transported into aerial tissue. Lytle *et al.* (1998), using X-ray spectroscopy, found that *Eichhornia crassipes* (water hyacinth), supplied with Cr(VI) in nutrient culture, accumulated non-toxic Cr(III) in root and shoot tissues. The reduction of Cr(VI) to Cr(III) appeared to occur in the fine lateral roots. Chromium (III) was subsequently translocated to leaf tissues. Extended X-ray absorption fine structure of chromium in leaf and petiole differed when compared to chromium in roots. In roots Cr(III) was hydrated by water, but in petiole and more so in leaf, a portion of the Cr(III) may be bound to oxalate ligands. This suggested that *E. crassipes* detoxified Cr(VI) upon root uptake and transported a portion of the detoxified chromium to leaf tissues. Chromium-rich crystalline structures were observed on the leaf surface.

1.1.6 Health effects of chromium

Four routes of exposure can be considered of interest with regard to chromium: (i) dermal absorption, (ii) ingestion, (iii) inhalation and (iv) ingestion secondary to inhalation. Chromium can act directly at the site of contact or can be absorbed into, or through human tissues.

There seem to be significant differences between chromium species and their health effects. Chromium (III) is considered an essential element in mammals, as it appears to regulate glucose tolerance (Friberg *et al.*, 1986). In addition, it appears to play an important role in the maintenance of vascular integrity. On the other hand, Cr(VI) is a strong oxidant which passes through cellular membranes at a rate many orders of magnitude faster than Cr(III) (Levis and Bianchi, 1982).

1.1.6.1 Effects of chromium from dermal exposure

Chromium (VI) can act as an oxidant directly on the skin surface or it can be absorbed through the skin, especially if the skin surface is damaged (WHO, 1988). The main effects reported are dermatosis (skin ulceration) and dermatitis (allergic skin sensitisation) (Bagdon and Hazen, 1991; Pedersen, 1982). Dermatitis is the most prominent reaction from the interaction of Cr with the skin (Nieboer and Jusys, 1988). Cr is generally recognised as the second most common skin allergen in the general population after nickel (Polak, 1983; Haines and Nieboer, 1988); in men it is the most frequent sensitiser (Haines and Nieboer, 1988).

The absorption of Cr(VI) into the blood system through the skin has been reported but not extensively investigated. Once absorbed into

the blood there seem to be various antioxidants, such as ascorbate and glutathione, which reduce Cr(VI) to Cr(III) (Levis and Bianchi, 1982).

Large doses of chromium in blood can result in acute kidney and liver damage (NIOSH, 1975; Langard 1982). Chronic liver and kidney damage due to long term exposure to Cr(VI) has also been reported (Mutti et al., 1979), although chronic low-level exposure to chromium does not appear to produce measurable renal damage (Nagaya *et al.*, 1994).

1.1.6.2 Effects of chromium caused by ingestion

Chromium can be incorporated into the gastrointestinal system by the direct ingestion of products containing chromium (water, food, soil, plants and animal products) or through particles cleared from the respiratory tract to the oesophagus. Chromium is present in food and feed plants, but in what form is not well characterised (Cary, 1982). Possible forms may be soluble Cr(III) organic compounds (*e.g.* chromium oxalate, Cr(III)-amino acid complexes) (Smith *et al.*, 1989; Nieboer and Jusys, 1988). Chromium (VI) can be ingested in contaminated water and particles.

If any Cr(VI) is ingested, it is believed that most of it will be quickly reduced in the acidic pH of the stomach, and only intakes that exceed the reducing capacity of the stomach would result in absorption of Cr(VI) across the gastrointestinal mucosa. At such large doses, Cr(VI) has resulted in toxicity to the blood, liver and kidney (Kaufmann *et al.*, 1970).

1.1.6.3 Effects of chromium after inhalation exposure

Absorption of Cr(VI) through the upper respiratory system produces several different health effects, mainly the ulceration or perforation of the nasal septum, the irritation of the upper airways and cancer of the respiratory system.

One of the key factors in the respiratory toxicology is the penetration of particulates into the lower lung airways. The largest particles that can pass the nose and throat are about 10 μm in diameter, but generally the greatest human health risks are posed by particles ranging from 0.2 to 2 μm in diameter (Langard, 1982), which can penetrate deep into the lungs. It has been suggested that more than 50 % of these particles can reach the gastrointestinal tract (Sheenan *et al.*, 1991). Thus chromium in the form of welding fumes, ore roasting fumes, and dust from precipitative and thermal chemical processes, all of which contain mostly PM10[1] particles, pose the greatest risk of inhalation into the lower lung airways.

Unlike most other toxins, absorption into the blood is not the principal health concern with chromates. Rather, the principal concern is the effect of chromates on the bronchi of the lung (IARC, 1990; Cohen *et al.*, 1993).

Respiratory cancer is the health effect of most concern and is the basis for the regulation of Cr(VI). There is also some indication that Cr(VI) may cause cancer of the upper airways and upper gastrointestinal tract, such as the oesophagus, larynx, trachea, and stomach (Alexeeff *et al.*, 1989). Although Cr(VI) appears to be a contact carcinogen to the respiratory system, it has not been

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[1] Particulate matter of aerodynamic diameter less than 10 μm (Duffus, 1998).

implicated in skin cancer, where there is far more frequent and intense contact than for any other part of the body. Also, Cr(VI) has not been reported to be carcinogenic to organs for which the only exposure is *via* the blood.

In several epidemiological studies, slightly elevated incidence of stomach cancer was reported (Alexeeff *et al.*, 1989). In these cases the route of exposure was inhalation, not ingestion, perhaps indicating that chromium reaches the stomach *via* clearance of the mucous membranes lining the airways. However, these results are not definitive, and it is not widely accepted that Cr(VI) is a carcinogen in the stomach (Lees, 1991).

Animal studies on the carcinogenicity of various Cr species, have generally suggested that water insoluble species, $CaCrO_4$ in particular (Lasking *et al.*, 1970; Levy *et al.*, 1986), are the causative agent of respiratory cancers.

Research on the carcinongenicity of Cr(VI) has focused on the fact that chromate ions quickly pass through cellular and nuclear membranes, while the trivalent species are many orders of magnitude slower (Lavelle, 1991). However, Cr(III) binds much more readily to DNA than chromate ions. Once in the cytoplasm, chromate ions can either pass the nuclear membrane and be reduced to Cr(III) or be reduced in the cytoplasm (Jenette, 1979). Because neither Cr(III) nor Cr(VI) react strongly with DNA, it is thought that the reduction of Cr(VI) to Cr(III), either in the cytoplasm nucleus or the blood, produces free radicals (OH^-, O, O_2^-, RS^-, R^- *etc.*) which in turn can bind to DNA (Wetterhahn, 1989). Indeed the DNA-protein cross links induced by chromate have been used as a biomarker for chromium exposure (Taioli, 1995.)

There is a vast literature documenting the mutagenic and cytogenic effects of various chromium compounds. Various laboratory studies have shown that Cr(VI) compounds cause many kinds of genetic damage (Cohen *et al.*, 1993).

1.1.7 Analysis of environmental chromium

The types of analysis that are used for the determination of chromium include total chromium and Cr(VI) analysis. The analysis of total chromium is less complex than the analysis of Cr(VI). There is a wealth of published methods for the determination of total chromium in a wide variety of matrices, but the most common approach is the use of hot acids that destroy chemical and physical bonds between chromium and the sample matrix and convert chromium into water soluble forms, leaving the bulk of non-targeted elements as solids that can be filtered out or oxidised to gases. USEPA SW-846 methods 3050 and 3051 (USEPA, 1996; 1998) use nitric acid, mixtures of nitric acid and hydrochloric acid, or *aqua regia* and are widely used and adequate for most solid waste and soils samples. Many aqueous samples containing significant quantities of particulate matter must also be digested in order to dissolve the sorbed chromium.

Total chromium in solution can be analysed by a variety of instruments. The most commonly used are electro-thermal (or graphite furnace) atomic absorption spectrometry (ETAAS, GFAAS), flame atomic absorption spectrometry (FAAS) or by inductively coupled plasma - optical emission spectrometry (ICP-OES) and inductively coupled plasma - mass spectrometry (ICP-MS).

The sampling procedures and the manipulation and storage of samples in the laboratory may result in chromium species interconversion, precipitation or dissolution (Beaubien *et al.*, 1994), affecting the results of the analysis and the interpretation of the possible hazards posed by chromium in environmental matrices. The analysis of aqueous samples requires little pre-treatment prior to analysis, but the acid digestion methods described above would be unsuitable for the speciation of chromium in solid matrices. Therefore, an alkaline digestion method must be used, to extract Cr(VI) into solution from solid samples, without reduction of the Cr(VI) or oxidation of Cr(III) species (USEPA, 1992a).

Chromium (VI) can be determined in water samples and soil extracts/digests by a colorimetric technique using 1,5-diphenylcarbazide as the complexing agent for chromate (USEPA, 1992b). Another method, introduced recently, employs speciated isotope dilution mass spectrometry (SIDMS), which enables the measurement of both Cr(VI) and Cr(III) in solution (USEPA, 1998).

1.1.8 Environmental risks

As mentioned before, Cr(VI) has been found to be toxic to a number of organisms when in aqueous phase. However, due to the dynamic interconversion of Cr(III) into Cr(VI) in aqueous environments, the availability of Cr(III) may lead to a health risk if this conversion occurs.

Chromium (VI) in soils may pose a risk of dermatitis and cancer. The risk of dermatitis can be associated with the degree to which Cr(VI) is absorbed into the skin, this depending on how much Cr(VI) can leach from soil and potential redox processes. Cancer risk may arise

from the inhalation of breathable particles. However, naturally occurring particles, such as abraded crustal materials, are unlikely to have a MMAD of less than 10 μm, and therefore are far too large to penetrate deeply into the lung. Furthermore, Cr(VI) may well be in water-soluble form, which is either entirely non-carcinogenic or only very slightly carcinogenic. Finally, if it is determined that water-soluble chromium attached to breathable particles presents a cancer risk, then given the potential for the oxidation of Cr(III) to Cr(VI) in some soils, Cr(III) may also be of concern.

The principal human health risk is the inhalation of water-insoluble industrial chromium breathable particles, for example released to the air directly by industrial processes, or as fugitive emissions from improperly stored or disposed of chromium materials.

1.1.9 Emerging needs for risk assessment and management - the basis for policy and regulation

The primary purpose of regulating chromium in the environment is to control and reduce the risk of lung cancer and dermatitis to the general public associated with exposure to Cr(VI). Because Cr (VI) is also toxic to some aquatic organisms and microorganisms used in wastewater treatment (Garcia *et al.*, 1994, Alkan *et al.*, 1996), it is also desirable to reduce Cr(VI) release into these systems.

The major problem with the regulation of chromium (VI) is the lack of reliable analytical procedures to extract Cr(VI) quantitatively and the interconversion of Cr species during sampling and extraction. In that context, policy-makers and regulators face several questions when they try to decide on risk reduction measures and safe thresholds (Kimbrough, 1999):

- if Cr(III) and Cr(VI) interconvert during analysis, what is the expected transformation in the environment?

- is the analytical extraction procedure representative of biological conditions and thus a reliable indicator of bioavailability?

- if the different Cr(VI) compounds have different physicochemical properties, do they have different toxicological effects?

- if Cr(III) and Cr(VI) interconvert and all Cr(VI) compounds are not equally hazardous, would the risk to the environment and human health be reduced by regulating Cr(VI)?

Risk analysis based on the determination of Cr(VI) as opposed to total chromium probably underestimates some risks and overestimates others. The use of total Cr(VI) overestimates most cancer risk assessments because it fails to distinguish water-soluble from insoluble chromium(VI) in non-aqueous matrices. An analysis based on Cr(VI) determination alone does not account for the total amount of Cr that can become water-soluble, thus such an analysis may underestimate the accompanying allergic or biota risk.

In order to properly assess and reduce the risks posed by chemical species in general, research needs include the improvement and use of statistical sampling protocols and statistical treatment of data, the refinement of analytical methods, the use of bioassays, information on bioavailability and pharmacokinetic behaviour *etc.* (Pollard *et al.*, 1996). Clearly, with respect to chromium, there is a need to

establish a scientifically defensible risk assessment process that considers its complexity in the environment and provides a more realistic assessment of human and ecosystem health risk (Kimbrough *et al.*, 1999).

1.2. Combining chemical and biological analysis to study bioavailability, environmental toxicity and ecotoxicity of Cr in COPR-contaminated environmental matrices

1.2.1 Bioavailability

From an ecotoxicological standpoint, to know the absolute concentration of a chemical present in a medium or compartment is not as important as to know the percentage that reaches organisms and the chemical species that does so. These factors contribute to the bioavailability of a substance, which in general depends on the chemical species and its physicochemical properties; the composition and characteristics of the environmental matrix; the possibility of uptake or the contact between substance and organism; and the physiological status of the organism.

The mobility, transport and partitioning of potentially toxic elements (PTEs) in the environment is a function of their chemical species, which in turn is controlled by their physicochemical properties and the physicochemical and biological properties of the systems where they are found (Kersten and Förstner, 1995). The term chemical species refers to the molecular forms of an element or a cluster of different elements in a given matrix (Berhard *et al.*, 1986).

It is common in environmental sciences to talk about speciation, a term which can have several definitions. Here, those quoted by Berhard *et al.* (1986) will be considered:

- The actual distribution among molecular level entities in a given matrix.

- The processes responsible for an observable species distribution.

- The analytical methods.

When dealing with soils, speciation may further be described by considering the type of bonding between an element and other components of soil, as well as from the standpoint of a process, such as plant nutrient uptake (available or unavailable forms) or mobility (readily leachable and slowly leachable) (Ritchie and Sposito, 1995).

Three main approaches can be used for the characterisation of PTEs speciation in contaminated soils:

- Geochemical theory with computer-based modelling.

- Understanding of the soil processes and conditions which control the reactions and transformations (chemical and biological) of metal species and hence influence metal mobility.

- Laboratory analyses of different metal fractions and forms in polluted soils, both from the chemical and biological standpoints.

There are remarkably few examples, if any, of ecosystem and soil studies that have used a combination of these three approaches in an attempt to provide more detailed interpretations of PTE processes in soils and a clearer picture of their short and long term fate.

Potentially toxic elements that may be bioavailable in soils are principally in soluble forms (soil solution and adsorbed on soil particles).

Soil properties that are likely to influence PTE reactions, transformations and mobility include: particle size distribution and particle surface area, bulk density, temperature, aeration and redox status, pH, ion exchange capacity, quantity and quality of organic matter, type and amount of Fe, Mn and Al oxides, and the type and amount of clay minerals.

According to Plant and Raiswell (1983), many metals are relatively more mobile under acidic, oxidising conditions and are retained very strongly under alkaline and reducing conditions.

Some of the main processes associated with potentially toxic elements in soils are:

- weathering of *in situ* parent material;

- dissolution and solubility of minerals and complexes, accompanied by precipitation and co-precipitation of inorganic insoluble species, such as carbonates and sulphides;

- uptake by plant roots and immobilisation by soil organisms;

- exchange onto cation exchange sites of clays or soil organic matter;

- specific chemisorption and adsorption/desorption on oxides and hydroxides of iron, aluminium and manganese;

- chelation and complexation by different fractions of soil organic matter,

- leaching of mobile ions and soluble organo-metallic chelates.

As discussed previously, chemical analyses cannot by themselves solve the problems associated with the distinction of "real" risks posed by chromium (or any other toxicant) in environmental samples. In natural systems, toxicity is not constrained by practicality, but it is manifested across all levels of biological organisation, involving compensatory reactions and interactive malfunctions more complex than a simple adverse effect. A holistic definition of toxicity accepts toxicity as a complex continuum of biochemical, physiological, whole organism, population and community responses among a broad diversity of living organisms (Luoma, 1995).

If it is accepted that toxicity is also organism-dependent, an approach that can be useful in the study of toxicants is a combination of chemical analysis with biological analysis (bioassays) in order to better assess the bioavailability and environmental toxicity and ecotoxicity of Cr.

1.2.2 Bioassays

Bioassays study the response of an organism(s) to contaminant(s). These involve many different biological mechanisms, both natural and genetically induced. Response can be assessed at any level of biological organisation through the use of single species, a battery of single species or communities exposed to the same analyte (Luoma, 1995). Bioassays can determine the concentration at which specific chemicals are toxic (bioassays, biomonitors, biosensors), the toxicity of an environmental compartment (bioindicators, biosensors) and/or clarify the processes that determine toxicity (biomarkers) (Johnston, 1995; Walker, 1995).

The degree of complexity of the bioassay depends on the purpose for which it is designed. Environmental toxicologists search for simple bioassay systems, which are less expensive and easier to standardise than field studies (Luoma, 1995). Ecotoxicologists[2] on the other hand, tend to use complex tests, which normally involve several species and take into account several factors influencing the toxicity of the chemical(s) under study (Chapman, 2002).

1.2.2.1 Multi-species and community bioassays

Multi-species bioassays are used to study community responses to toxicant exposures. These range from a microcosm in a beaker to a

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[2] The domain of toxicology in general includes understanding of the types of effects caused by chemicals, the biochemical and physiological processes responsible for those effects, the relative sensitivities of different types of organisms to chemical exposures, and the relative toxicities of different chemicals and chemical classes. Ecotoxicology comprises the integration of ecology and toxicology. Its objective is to understand and predict effects of chemicals on natural communities under realistic exposure conditions. Environmental toxicologists generally test individual species rather than combined species (Chapman, 2002).

manipulated lake. A few species can be introduced to the system or a natural community can be enclosed, manipulated or transplanted (Ravera, 1989). The choice of scale and complexity is related to economy, control, ease of interpretation and direct applicability to nature.

The most complex approach is ecosystem manipulation (lake, pond, stream systems). These provide the best simulations of whole system reactions to a toxicant, although the cost is that many important variables can be difficult to control.

Changes in species composition of a community, or even loss of a single species, can potentially cause a cascade of indirect changes through a food web. A problem of confining communities for bioassays is that it can limit predation and biological interactions that are important in open systems.

1.2.2.2 Single species bioassays

Single species bioassays test sub-lethal stress or survival in adults or in individuals from a sub-adult life stage. These are widely used in environmental toxicology, perhaps because they balance the advantages and disadvantages of other approaches. They are relatively easy to manage, control and standardise. Standardised methodologies allow reliable and repeatable results and relatively rapid data collection (Maltby and Calow, 1990).

A variety of species has been employed in single-species bioassays. Well-designed single-species bioassays can unambiguously test hypotheses or be used to develop generalisations about causes of toxicity.

1.2.2.3 Level of biological organisation

Every bioassay involves choices about the level of biological organisation to study, exposure time, exposure concentration, exposure route, and how representative of ecosystems are the species or system employed in the study.

The level of biological organisation chosen for a bioassay depends upon its purpose. In general, factors taken into account are the sensitivity of the test, the ease with which a response can be detected, relevance to natural environmental conditions and usefulness to managers. Sensitivity and ease of detection are often inversely related to usefulness and relevance. Biochemical and physiological responses are sensitive and relatively easy to detect, however their relevance to higher ecosystem changes may not be clear. Single species bioassays directly approach questions about the response of individuals, but they are not relevant to more complex ecosystem processes, including species interactions, population dynamics, interrelations of organisms with biogeochemical cycles or other higher-order responses (Cairns, 1983). Population and community change are the response of greatest relevance to environmental managers and the most direct indicators of degradation. Generally, responses at these levels of organisation are more difficult to study.

1.2.2.4 Exposure time and concentration

The effects of exposure time and exposure concentration are related, *e.g.* test species respond to progressively lower metal concentrations as exposure time increases (Luoma, 1995). The shorter the exposure time, the higher is the concentration necessary to trigger a response.

Chronic exposures in nature can persist through generations of resident species; thus, chronic bioassays that rigorously simulate nature should include exposures over multiple life cycles. Whole organism bioassays should extend at least for the full life span (generation time) of the adult or life stage being studied. Tests with short exposure times, relative to the life history of the test species, can underestimate the chronic toxicity of an element by orders of magnitude (Luoma and Carter, 1991).

Simulations of chronic exposures in nature are easiest with species with short generation times; microorganisms are extremely useful in this sense, as they reproduce and develop fast enough to allow for the testing of several generations in a manageable time.

1.2.2.5 Route of exposure

The route of exposure may also affect interpretations. Presentation of the toxicant in solutions has long been the most common bioassay approach. Control of metal concentration, geochemistry, reducing/oxidising conditions and the build up of metabolites is easier when the experiment is conducted in solution. Nevertheless, the behaviour of toxicants in solid environmental systems, such as sediments and soils, is more complex than simple solution systems and the interactions between the toxicant and other matrix components should be taken into account.

1.2.3 Biosensors

Biosensors measure the interaction of chemicals with biological systems through a biomolecular recognition capability. A biosensor

is made from a biological sensing element coupled to a signal transducer (Rogers and Gerlach, 1996). The sensing element of the biosensor can be placed in a distinctive group: behavioural, catalytic and non catalytic. The behavioural group consists of living higher organisms (*e.g.* fish, mussels, *etc.*); the catalytic group includes enzymes, microorganisms and tissues; the non-catalytic or affinity class comprises antibodies, receptors and nucleic acids (Van der Lelie *et al.*, 1994). The transducer may be electrochemical, optical or acoustic.

Biosensors are distinguished from other bioassays, such as immunoasays and enzyme assays, because the analyte tracers or catalytic products can be directly measured, normally during a single-step operation (Rogers and Gerlach, 1996).

There has been extensive research to develop biosensors that can be used in the form of small portable analysers for *in situ* monitoring of air, soil and water quality. Dedicated biosensors can also be developed for off-line monitoring applications in the laboratory and for on-line applications for the monitoring of contaminants (van der Lelie *et al.*, 1994).

Examples of biosensors developed for the analysis of environmental samples in the laboratory are the microbial luminescent biosensors.

1.2.3.1 Luminescent microbial biosensors and principles of bioluminescence

Bioluminescence is the process of visible light emission in living organisms mediated by an enzyme catalyst. Bioluminescent organisms are widely distributed in nature and comprise a

1.2.3.1 Luminescent microbial biosensors and principles of bioluminescence

Bioluminescence is the process of visible light emission in living organisms mediated by an enzyme catalyst. Bioluminescent organisms are widely distributed in nature and comprise a remarkably diverse set of species, including bacteria, dinoflagellates, fungi, fish, insects, shrimps and squids (Meighen, 1993).

Luminous bacteria are the most abundant and widely distributed of the light-emitting organisms and are found in marine, fresh water and terrestrial environments. Almost all luminous bacteria have been classified into three Genera: *Vibrio*, *Photobacterium* and *Xenorhabdus*. The light-emitting bacteria that have been investigated in most detail are *Vibrio harveyi*, *Vibrio fischeri*, *Photobacterium phosphoreum*, *Photobacterium leiognathi* and *Xenorhabdus luminescens* (Meighen, 1991).

The recognition by researchers of the ease and sensitivity of detection of light emission has led to widespread application of the genes coding for light production as reporters of gene expression and regulation (Corbisier *et al.*, 1996), as well as sensors for metabolic function in the cells of diverse prokaryotic as well as eukaryotic organisms.

The production of light involves the oxidation of a long-chain fatty aldehyde and reduced flavin mononucleotide ($FMNH_2$) which results in the emission of a blue-green light at around 490 nm, along with the production of oxidised flavin and a long-chain fatty acid (Hastings *et al.*, 1985):

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + \text{light (490 nm)}$$

The natural aldehyde for the bioluminescent reaction is believed to be tetradecanal. However differences in aldehyde specificity do exist among different bacterial luciferases (Meighen, 1991).

The genetic information that encodes the enzymes required for the bioluminescent reaction is contained in the structural *lux* genes. These have been cloned from several luminescent bacteria and they have a similar genetic organisation within the *lux* operon. The production of luciferases, which contain two non-identical subunits α and β are encoded in the *lux*A and *lux*B genes. The aldehyde substrate required by the luminescent reaction is produced from a long chain fatty acid by a multienzyme fatty acid reductase complex containing three proteins – a reductase, a transferase and a synthetase. The genes *lux*C, *lux*D and *lux*E encode the production of these three proteins. The former two are located upstream of the luciferases genes and *lux*E is located downstream; and additional gene *lux*F is located between *lux*B and *lux*E in certain *Photobacterium* species. In general the *lux* operon is denoted as *lux*CDABE with transcription occurring from left to right.

Nowadays there is sufficient progress in the cloning and characterisation of the *lux* structural genes to allow exploitation of their biotechnological potential. In 1982, the isolation of *V. harveyi* luciferases genes and their subsequent expression in *Escherichia coli* (*E. coli*) (Belas *et al.*, 1982) marked the first conversion of a non-bioluminescent cell type with the *lux* phenotype. The *lux* genes have been transferred using plasmids into different bacteria, including Gram-positive and Gram-negative bacteria, plant pathogens and marine bacteria (Meighen, 1991).

1.2.3.2 Applications of luminescent biosensors in environmental toxicology

The first commercial kit to take advantage of luminescent bacteria, the Microtox® system, uses *Photobacterium phosphoreum*. Toxic effects of compounds are correlated with the inhibition of bioluminescence. This is based on the principle that *in vivo* bioluminescence requires a functional intracellular biochemistry and, as such, inhibition of luminescence can represent an accurate reflection of impaired metabolism of reduced cellular viability. Therefore dead cells produce no light, while sub-lethally damaged cells are reduced in luminescence, in such a way that the toxic effect of specific chemicals can be evaluated by measuring the light output of the culture. Several luminescent biosensors are based on this principle (Chatterjee and Meighen, 1995).

Natural luminescent bacteria such as those used by the Microtox® system have been used to assess the toxicity of contaminated solutions, water and soil pore water samples, testing the toxicity of potentially toxic elements such as mercury (Ribo, *et al.*, 1989), Cr(III) and Cr(VI) (Garcia *et al.*, 1994; Villaescusa, 1997) and organic compounds (Bundy *et al.*, 1999).

Some of the genetically modified "dark" microorganisms, in which the *lux* operon has been inserted include *Pseudomonas fluorescence*, *Rhizobium leguminosarium* biovar *trifolii* and *E. coli*, which have been used as biosensors to assay the toxicity of potentially toxic elements such as Zn, Cd, Cr and Ni in solution and contaminated soil porewaters (Campbell *et al.*, 2000; Chaudri *et al.*, 2000; Paton *et al.*, 1997, Vulkan *et al.*, 2000).

A second class of luminescent biosensors is specifically designed to detect certain analytes by including promoter genes that are subject to environmental regulation with the genes required for luminescence. In most of the cases, microorganisms produce light in the presence of the analytes. Metal-*lux* fusions that have been constructed and studied include those for the detection of arsenite, cadmium, cobalt, chromate, lead, nickel and thallium (Corbisier *et al.*, 1993, 1994, 1996) some being able to distinguish between metal species, *e.g.* Cr(III) and Cr(VI) (Corbisier, 1999).

1.2.4 Community assays. Signature lipid biomarker analysis and community-level physiological profiles: tools to study the effects of chemicals on soil community structure

Despite the critical geochemical roles that microorganisms play in biosphere maintenance, very little is known of how they function in soils, sediments and waters and how they are affected by contaminants. Microorganisms are undoubtedly primary agents of geochemical change. Their growth and survival drive the geochemical cycling of the elements, detoxify systems from contaminants, make essential nutrients present in the biomass[3] of one generation available to the next, and maintain the conditions required by other inhabitants of the biosphere. Processes carried out by microorganisms in soils, sediments, oceans, lakes and groundwater have a major impact on environmental quality, agriculture, and global climate change (Schlesinger, 1991).

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[3] The total living mass in a defined segment of an ecosystem expressed as the living weight per unit area or mass. Soil microbial biomass is often used as an indication of potential microbial activity level in soil.

Complete characterisation of microbial communities in soils is not possible because biomass occupies just 0.001% of the soil volume (Madsen, 1998). On the other hand, the classical methods of isolation and culture of microorganisms used in clinical studies do not work when applied to the environment, as less than 1% of what can be detected in stained microscopic preparations can be cultured (White, 1995). Staining microorganisms in soils can be difficult as many are attached to soil granules and can be hidden. Agents that release attached microorganisms are often selective and do not release them quantitatively. Furthermore, the morphology of the microorganisms does not often reflect the function or activity, so very little insight into the community structure or nutritional status is possible.

Studies of soil microbial properties have generally been based on the process level, studying parameters such as biomass, respiration rates, and enzyme activities, which provide an important understanding of gross microbial processes and their potential role in soil health, but they give little information regarding qualitative community-level changes, because any given microbial process may be carried out by diverse *taxa*.

Community-level microbial interactions are complex, individual species relying on the presence, function, and interaction of many other species. Therefore, quantitative and qualitative changes in the composition of soil microbial communities may serve as important and sensitive indicators of both short- and long-term changes in soil health. The analysis of soil microbial communities should involve not only determinations of microbial biomass and diversity, but also determinations of microbial growth, distribution and function.

The approach to analysing soil microbial communities has changed in the last decade, with new methods and approaches allowing for better assessments of microbial diversity, with the conventional analysis by plating being complemented or substituted by other techniques.

1.2.4.1 Community-level physiological profiles

The traditional method of community-level physiological profiles (CLPP) involves the dilution and inoculation of soil suspensions in plates containing different carbon sources. The utilisation of those carbon sources is detected by the reduction of an indicator by spectrophotometry (Hill *et al.*, 2000). This technique takes advantage of the traditional methods of bacterial taxonomy in which bacterial species are identified based on their utilization of different carbon sources. Community-level physiological profiles have been facilitated by the use of a commercial taxonomic system, known as the BIOLOG®, which has been extensively used for the analysis of soil microbial communities (Winding, 1994; 1995; Garland, 1996b). This BIOLOG® system is based on the utilization of a suite of 95 different carbon sources that have been described previously (Garland and Mills, 1991). The pattern of substrates that are oxidized can be compared among different soil samples from a series of times or locations as an indication of differences in the physiological functions of microbial communities. Multivariate statistical techniques are generally used to analyse the substrate utilization profile data (Hitzl *et al.*, 1997). There are a number of important drawbacks to this technique, such as the difficulty of standardization of the inoculum, the predominance of species which might be more prone to grow on particular substrates, the period of microbial growth within the well, and also that the substrates found

in commercially available BIOLOG® plates are not necessarily ecologically relevant and most likely do not reflect the diversity of substrates found in the environment (Campbell *et al.*, 1997a).

While the BIOLOG® CLPP may provide information useful for assessments of soil microbial community diversity, the method still suffers from the same problems encountered with culture plating methods, making data interpretation problematic.

Campbell *et al.* (2001) have developed a method based on the CLPP principle that does not rely on culturing methods. Soil samples are directly mixed with carbon sources and an indicator registers the utilisation of carbon sources, the change in colour being measured by spectrophotometry. This technique has potential applications, as samples do not need to be manipulated excessively and community structure can be studied 'intact'.

1.2.4.2 Signature lipid biomarker analysis

Because of the inherent limitations of culture-based methods, soil microbial ecologists are working increasingly with culture-independent methods of community analysis. Culture-independent methods can give important information on the composition of soil communities by using the extraction, quantification and identification of molecules from soil that are specific to certain microorganisms or microbial groups. Advanced fluorescence microscopic techniques can also be used for this purpose. The molecules used in this context include phospholipid fatty acids and nucleic acids (Morgan and Winstanley, 1997).

molecules used in this context include phospholipid fatty acids and nucleic acids (Morgan and Winstanley, 1997).

Lipids are integral components of the membrane of all cells and play a role as storage materials and they are easily recovered by extraction in organic solvents (Tunlid and White, 1992; Zelles and Bai, 1993). Signature lipid biomarker analysis (SLB), the analysis of the cellular lipids, provides a quantitative insight into three important attributes of microbial communities (Figure 1.2): viable biomass, community structure and nutritional status.

Viable biomass[4]. Viable microorganisms have an intact membrane that contains phospholipids and phospholipid fatty acids (PLFAs). The cellular enzymes hydrolyse the phosphate group within minutes to hours of the cell death (White *et al.*, 1979). The lipid remaining is a diglyceride (DG). The resulting DG contains the same signature fatty acids as the phospholipids; in that way the comparison of PLFAs (viable microorganism) to DGFAs (non-viable microorganisms) can be made.

Community structure. The analysis with SLB provides a quantitative definition of the microbial community structure. Specific groups of microorganisms often contain unusual lipids (Tunlid and White, 1991). For example, there are specific PLFAs, which are prominent in the hydrogenase-containing *Desulfovibrio* sulphate-reducing bacteria, whereas the *Desulfobacter* type of sulphate-reducing bacteria contains distinctly different PLFAs (White, 1995). Groups of bacteria have been isolated from marine sediments such as methanotrophs, sulphate reducers and *Thiobacilli* spp with specific types of fatty acids (Guckert *et al.*, 1985).

since polyenoic fatty acids, with few exceptions, only exist in eukaryotes, while a number of other fatty acids are found almost exclusively in bacteria (Frostegård *et al.*, 1993). Gram-positive bacteria have been separated from Gram-negative bacteria on account of their PLFAs, the former seeming to have a high content of branched chain fatty acids while the latter seem to have a higher content of monoenoic and cyclopropane fatty acids (Federle, 1986).

Table 1.2 includes PLFAs that have been identified in particular microorganism groups. Fatty acid analysis has long been used for bacterial taxonomy, in which specific fatty acid methyl esters (FAMEs) have been used as an accepted taxonomic discriminator for species identification (Hill *et al.*, 2000).

Table 1.2 Ester-linked PLFA biomarkers associated with specific taxonomic groups. Adapted from Chapman *et al.* (2000).

Taxonomic Group	PLFA Biomarker*
Fungi and other eukaryotes (protazoa)	Polyunsaturated fatty acids 18:3ω6, 18:2ω9; 20:4. Even, straight-chain saturated fatty acids.
Fungi	18:2ω6,9.
Actinomycetes	Methyl branching at the δ10 position, *e.g.* 10Me18:0.
Gram-positive bacteria	Branched chain fatty acids (*iso*, *anteiso*): including a15:0, i15:0, i16:0, i16:1, 10Me16, 10Me17, a17:0, i17:0. ω9 monounsaturated fatty acids.
Gram-negative bacteria	ω7 monounsaturated fatty acids, including 16:1ω7c, 18:1ω7. 16:1ω5.
Sulphate reducers	10Me16:0, 17:1ω6, br17:1, i17:1ω7.
Methanotrophs (Type I)	16-carbon monounsaturated fatty acids, including 16:1ω5c, 16:1ω8c, 16:1ω8t.
Methanotrophs (Type II)	18:1ω8c, 18:1ω8t, 18:1ω6c.

*Explanation of nomenclature given in Section 5.6.2

Nutritional status. Specific patterns of PLFAs can indicate physiological stress (Guckert, 1986). Exposure to toxic environments can lead to mini-cell formation and a relative increase in trans-monoenoic PLFAs compared to the *cis* isomers. In the same way, prolonged starvation or exposure to toxics such as phenols or organic solvents induce some Gram-negative bacteria to form *trans*

PLFA. Prolonged exposure to conditions inducing stationary growth phase cause the formation of cyclopropane PLFAs.

Phospholipid fatty acids are easily extracted from microbial cells in soil (Tunlid and White, 1992; Zelles and Bai, 1993), allowing access to a greater proportion of the microbial community resident in soil than would otherwise be accessed during culture-dependent methods of analysis. The presence and abundance of these signature fatty acids in soil reveals the presence and abundance of particular organisms or groups of organisms in which those signatures can be found.

Phospholipid fatty acids analysis has been used as a culture-independent method of assessing the structure of soil microbial communities and determining gross changes that accompany soil disturbances such as land use (Yao *et al.*, 2000; Zelles *et al.*, 1992), pollution (Frostegård *et al.*, 1993), fumigation (Macalady *et al.*, 1998), and changes in soil quality (Bardgett *et al.*, 1996; Bossio *et al.*, 1998; Reichardt *et al.*, 1997; Petersen *et al.*, 1998).

In summary, there are three major ways in which PLFA data from soil samples can be used:

- The entire PLFA profile can be used as a "fingerprint" of the whole soil community.

- PLFAs can also be used to indicate specific subgroups within the community.

- To indicate stress, through changes in the ratio of saturated to unsaturated fatty acids, ratio of *trans*- to *cis*-monoenoic unsaturated fatty acids, and the proportion of cyclopropyl fatty

acids. These and another changes can be good indicatives of toxicity of contaminants.

It may then be possible to define "soil quality" based on the viable biomass, community structure and nutritional/physiological status of the soil microbiota and make valuable predictions about crop yield or bioremediation effectiveness (White, 1995).

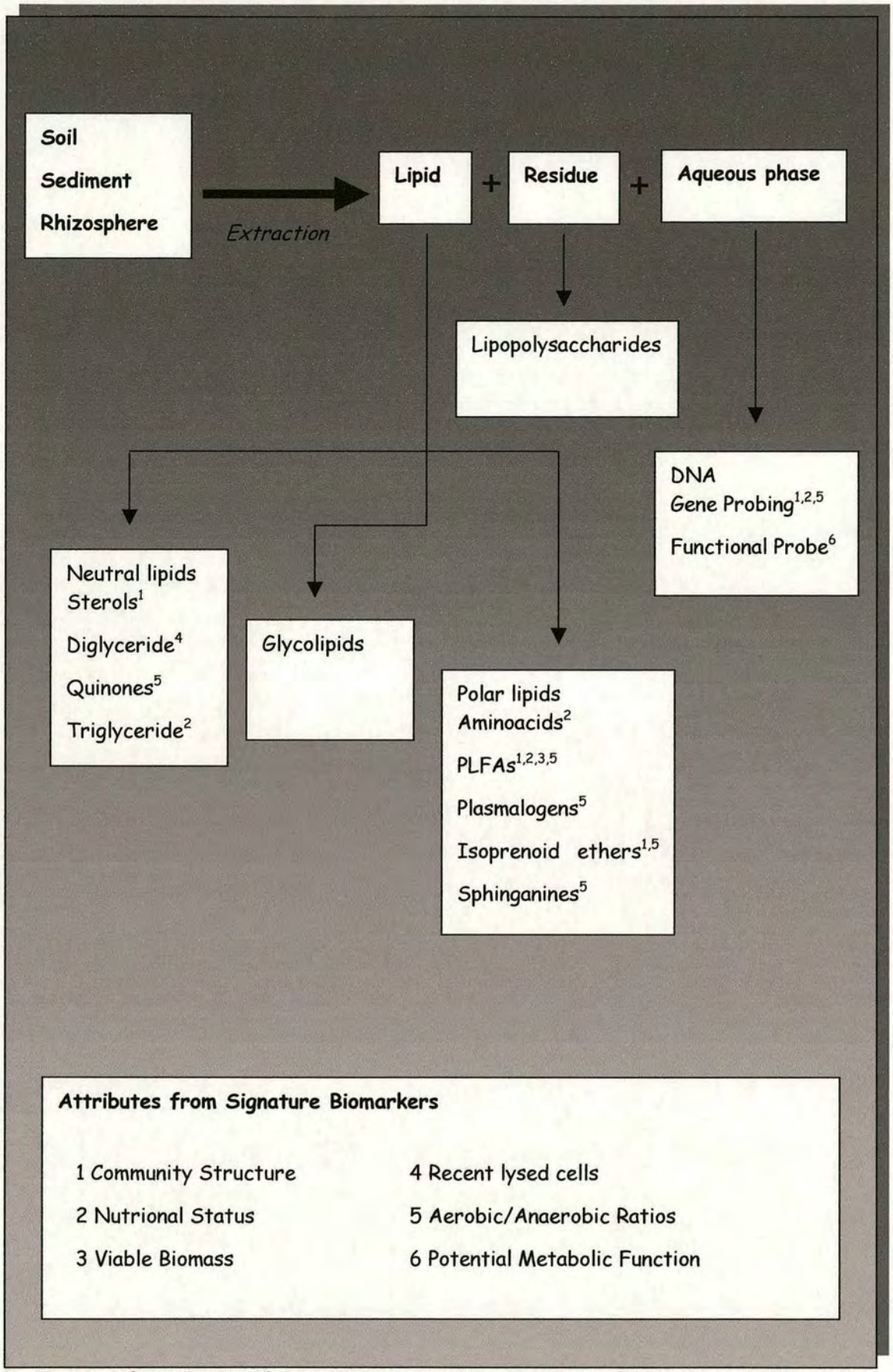

Figure 1.2 Attributes of microbial communities based on Signature Lipid Biomarker Analysis (Lipids and DNA). Adapted from White, 1995.

1.3 Urban soils and the chromium problem in SE Glasgow and South Lanarkshire

1.3.1 Urban soils

A useful definition of urban soil is that it is a soil material having a non-agricultural use, being a man-made surface layer more than 50 cm thick, that has been produced by mixing, filling, or by contamination of land surface in urban and suburban areas (Bockheim, 1974). There are several differences between soils in the urban areas and those in rural environments (Craul, 1985) and, so far, none of the existent soil classification schemes fulfil all the requirements for a proper urban soil classification (Hollis, 1991). There have been several attempts to extend the application of soils classification schemes into areas of high human activity but they still contain omissions or undeveloped concepts (Fanning *et al.*, 1978; Keleberda and Drugov, 1983;).

The characteristics of urban soils depend both on the parent material from which they were formed and the physical and chemical disturbances they have undergone over time, having a wide spectrum in terms of composition and degree of development. The production of urban waste materials began to increase in amount and complexity as industrial activities diversified and cities developed. Among the wastes which enter soils in urban areas can be found among others: building materials, chemical waste materials (Bridges, 1991), waste from metalliferous industries (Bridges, 1984), organic wastes (Heeps and Pike, 1980; Thomas, 1992; Campbell *et al.*, 1997b), waste materials from power generation plants (Bridges, 1991), from gasworks (DoE, 1979), waste

and spilled oils, wastes from scrapyards and from disposal sites (Bridges, 1987).

Urban soils are quite complex and highly disturbed systems, which need a different consideration to rural soils from the analytical, restoration and risk assessment points of view. The are several issues related to urban soils which require further research, among them: gathering inventories of polluted land, development of standardised analytical techniques, understanding of chemical speciation, study of the bioavailability and ecotoxicity of pollutants and their combined effects.

1.3.2 The chromium problem in SE Glasgow and South Lanarkshire

Approximately 2 million tonnes of chromite ore processing residue (COPR) have been used as landfill in several urban sites in SE Glasgow and South Lanarkshire (Farmer *et al.*, 1999). The COPR was a result of the lime-based chromate extraction process used by the White's chemical works from around 1830 until its closure in 1968. This has resulted in high levels of chromium contamination in places that, in some cases, are still used by the local community. The chromium content of the residue is 3-4 % w/w. Due to the high alkaline conditions (pH 9-12), Cr(VI) (in the form of chromate) is found in concentrations as high as 1 % w/w. The distribution and depth of the COPR vary and this may significantly influence the potential risks and options for remediation in the area (Licona Manzur *et al.*, 2001).

1.4 Conclusions and Aims

The potential risks posed by contaminated urban soils amidst the increasing need to determine threats to human health and the environment, as well the reclamation of land itself, have created the need to develop analytical tools for investigating the toxicity of contaminated sites and for monitoring the success of remediation approaches.

As the bioavailability of contaminants depends not only on the chemical properties of the contaminant, but also on complex processes *in situ* and the target organism, it has been suggested that the best way to study the toxicity of contaminants in soils might be *via* the use of combined chemical and biological analysis.

The dependence of Cr toxicity upon its speciation has had to be recognised in its regulation, and there is a need both to develop new approaches to study Cr toxicity *in situ* and to re-examine current regulatory limits.

This work aimed to answer some of the questions related to the bioavailability and (eco)toxicity of Cr using chemical and biological analysis, and to examine the usefulness of such an approach in the investigation of the (eco)toxicity of contaminated environments. Through the use of chemical analysis, single species bioassays and microbial community bioassays, the determination of possible hazards associated with contaminated sites and the monitoring of remediation techniques might be facilitated.

The specific objectives of the work were as follows:

- To investigate the effect of Cr(VI) in synthetic solutions on the single species bioassay *E. coli* pUCD607 at different pHs and to obtain concentration-response curves that could serve as a reference for comparison with environmental samples. In addition, to study possible factors contributing to the observed effects.

- To investigate the use of the single species bioassay *E. coli* pUCD607 in comparing the toxicity of Cr-contaminated environmental samples and to compare the results with the concentration-response curves obtained for synthetic solutions. In addition, to investigate the possible factors contributing to toxicity and to compare them with those identified for synthetic solutions.

- To study (under controlled conditions) the effects of added Cr(VI) on soil microbial community structure and their community level physiological profiles in soils with either different Cr(VI) adsorption capacity or with the same Cr(VI) adsorption capacity but different microbial community.

- To compare the direct effects (i.e in the absence of plants) of Cr(VI) from synthetic solutions on acute, *ex situ* single species bioassays with those on chronic, *in situ* bioassays (community structure and physiological profiles) in contaminated soils.

- To study the effects of Cr(VI) in leachates from Cr-contaminated soils on the microbial community structure and physiological profiles of soils in the presence of growing plants.

- To compare the sensitivity of different biological assays and suggest a possible integrated approach to studying the toxicity of contaminated sites.

Chapter 2

Analytical Methods

During this work several chemical and biological methods were used to investigate the (eco)toxicity of chromium. Those common to the complete work are described in this chapter, while specific methodology (*e.g.* sampling) is described in the individual chapters. The methods outlined in this chapter are divided into i) physicochemical, ii) chemical extractions and analyses, and iii) biological analyses.

2.1 Physicochemical methods

2.1.1 Soil water content by the thermogravimetric method

Principle

The determination of water content in a soil is fundamental to an investigation of the properties of the soil and its interactions with ecosystems. Measurements of this parameter may be undertaken in the laboratory or in the field. The required accuracy and precision determine the methodology that must be employed. Currently, three methods are used: thermogravimetry, neutron thermalisation, and a group of techniques based on measurement of soil dielectric properties (Gardner *et al.*, 2000).

The thermogravimetric method is perhaps the most widely used in the laboratory and is the only true direct method. It is based on the definition of soil water content, *i.e.* the water that may be evaporated from a soil by heating to between 100 and 110°C (usually at 105°C) until there is no further weight loss (ISO, 1993; USEPA, 1986). Soil water content may be expressed on either a mass (kg water per kg dry soil), or a volumetric (m^3 water per m^3 of bulk soil volume) basis. In either case the value is a dimensionless fraction and can be multiplied by 100 to express it as a percentage [Topp, 1993].

Potential problems associated with this method include the following:

- When investigating organic soils, inaccuracy in water content determination may occur due to the oxidation and

decomposition of organic matter at 105°C, causing weight loss other than that due to water evaporation

- In certain soils, volatilisation of substances other than water may occur at temperatures below 105°C. Lower drying temperatures may be considered when working with soils where this occurs but can lead to determination of significantly lower water contents

There is not an universal drying time, as it is influenced by the efficiency and size of the oven and the number and size of samples (Reynolds, 1970)

Apparatus and materials

- Convection oven at constant temperature (105°).
- Borosilicate 100 ml beakers or petri dishes.
- Three decimal place balance.
- Desiccator with self-indicator silica gel (Merck, UK).

Procedure

Duplicate determinations were carried out for each sample. Empty beakers were weighed to the nearest three decimal places and placed in a convection oven at 105°C. The weight of the beakers was measured at equal time intervals, until it was constant[1]. The last reading was recorded (*B0*).

[1] Constant mass is defined as that reached when the change in a sample's weight, after drying for a further 4 hours, does not exceed 0.1% of the weight at the start of the four hours (ISO, 1993).

Approximately 2 g of fresh soil (<2 mm) was weighed into the tared beakers to the nearest three decimal places (*FS*). Beakers were placed back into the oven and dried overnight[2] at 105°C. They were then transferred to a desiccator containing self-indicating silica gel, left to cool down and the total weight recorded (*DSB*]. The water content, expressed as a percentage (*%wc*) was calculated using the following formula:

$$\%wc = \frac{(FS) - (DSB - B0)}{(DSB - BO)} \times 100 \qquad \textbf{equation 2.1}$$

2.1.2 Water Holding Capacity

Principle

The water holding capacity (WHC), as used in this work, is a measure of the maximum amount of water that a soil can hold after being irrigated and under free drainage conditions. It depends on the soil particle size and therefore on the pore space available for water to be retained on it. The moisture content of a soil can be related to a percentage of its WHC. Under laboratory conditions, the WHC can be used to standardise different soils, *i.e.* to 70% WHC to extract comparable soil solutions.

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[2] With the samples used in this project a constant mass was attained after 12 hours

Apparatus and materials

- Glass funnel (15 cm diameter) fitted with a 5 cm piece of silicon tubing at the end
- Watch glass (15 cm diameter)
- Glass wool
- Plastic measuring cylinders (100 mls)
- Clamp
- Metal stand

Procedure

The water content of fresh soil samples was measured by the thermogravimetric method to determine water already present in the sample.

The glass funnel was supported on the metal stand and a clamp was placed (and closed) on the silicon tubing. Glass wool was fitted into the funnel, covering approximately 1 cm of the funnel neck.

The fresh soil sample (50 g) was placed into the funnel and carefully levelled, without compacting. Deionised water (100 ml) was added to the funnel and the watch glass was placed on the funnel to prevent evaporation. After 30 minutes the clamp was opened and the water was left to drain. After further 30 minutes the clamp was closed and the water collected in the measuring cylinder was measured.

Calculations

The water holding capacity of the soil, according to this method, is defined as the water retained after irrigation plus the water already present in the soil as moisture:

$$WHC = \frac{\%wc}{100} + wrds$$ **equation 2.2**

where % wc = Water content percentage and wrds = g of water retained per g dry soil. To calculate WHC the following procedure was used:

The water content according to the thermogravimetric method (2.1.1) is given by:

$$\%wc = \frac{(B) - (C - A)}{(C - A)} \times 100$$

The water content of soil samples and the amount of dry soil content related to the fresh soil weight was calculated from data obtained for the water content measurement according to:

$$D = \frac{(B) - (C - A)}{B}$$ ***equation 2.3***

$$E = 1 - D$$ ***equation 2.4***

where:

A= weight of empty beaker
B= weight of fresh soil
C = weight of dry soil plus beaker
D = g H_2O/g of fresh soil
E = g dry soil/g fresh soil

The moisture content relates equations 2.2 and 2.3, according to:

$$\%wc = \frac{D}{E} \times 100$$ **equation 2.5**

The amount of dry soil in the sample was calculated according to:

$$F = B \times E$$ **equation 2.6**

where:
F= g of dry soil in sample

Using the results from equation 2.5 is possible to calculate the water retained by the soil sample on a dry weight basis:

$$wrds = \frac{(100 - G)}{F}$$ **equation 2.7**

where:
wrds = g of water retained per g dry soil
G = weight of water collected in the measuring cylinder (assuming that density of water is 1)

As the particle distribution affects the amount of water held by the soil, the size fraction of the soil sample used to obtain the WHC must be specified when quoting the WHC.

2.1.3 Soil pH

Principle

The term "soil pH" refers to the pH value of a soil-water system which may vary in composition (Hess, 1971). Soil pH is affected by

many factors, including nature and type of inorganic and organic matter; the amount and type of exchangeable cations and anions; the ratio of soil to solution used in the measurement; salt or electrolyte content; and CO_2 content (McLean, 1982). The acidity, neutrality, or basicity of a soil influences the solubility of various compounds; the relative ion bonding to exchange sites; and microbial activities. Depending on the predominant clay type, the soil pH may be used as a relative indicator of base saturation. Soil pH is also a critical factor in the availability of most essential elements for plants.

Several methods can be used to determine soil pH, *e.g.*, soil in deionised water at different ratios; soil in 0.01M $CaCl_2$; and soil paste. In general, the greater the proportion of water the higher the pH (Hesse, 1971). For this reason it is important, when comparing different soils, that the same procedure is used at all times. In this case the pH has been measured in deionised water with a soil:water ratio of 1:2.

Available standard method for soil pH measurement: USEPA 9045c (USEPA, 1995).

Apparatus and materials

- Digital pH meter, Jenway 3305 (Jenway UK)
- BDH Gelplas (General purpose combination) electrode
- 30 ml universal containers (Merck, IK)
- Flask shaker (Stuart Scientific, SF1)

Reagents

- Deionised water

- Buffers: pH 4.0 and 7.0 from commercial tablets, pH 12.07 made from a $Ca(OH)_2$ saturated solution.

Procedure

The pH meter was calibrated using a pH 7.0 buffer and either pH 4.0 or pH 12.07 buffers as endpoints. After calibration the electrode was rinsed with deionised water. Sub-samples of 5 g (±0.01 g) of fresh soil were weighed into labelled polypropylene tubes, 10 ml of deionised water were added and tubes were closed tightly. Tubes were shaken for 30 min. and left to stand for further 10 min., whereupon the pH was measured. The electrode was rinsed with deionised water between samples.

2.1.4 Soil ash and organic matter content by loss-on-ignition

Principle

The ash content of organic soils is an important component of the soil matrix, which reflects the degree of mineral nutrient enrichment (Farnham and Finney, 1965).

There are basically two approaches to the determination of the ash content (or inorganic fraction) of a soil: dry ashing methods and wet ashing methods. Dry ashing methods have been employed widely for determining the ash content of organic soils and estimating the organic matter content of non-calcareous and forest soils, sediments and leaves (Ball, 1965; David, 1988; Sutherland, 1998; Fagbenrro *et al.*, 1999).

Dry ashing methods involve the removal of organic matter by combustion of the sample at temperatures between 375 and 600°C in a temperature-regulated muffle furnace for 12 to 15 hours. The substance remaining after ignition is the ash and includes mineral

impurities such as sand (Karam, 1993). Inaccuracies can arise both from volatilisation of metals and retention of metals in an insoluble form in the container (usually a crucible or high-temperature resistant beaker).

Apparatus and materials

- Muffle furnace
- Borosilicate beakers or crucibles
- Three decimal place balance
- Desiccator with self-indicating silica gel

Procedure

Beakers were weighed to the nearest three decimal places and placed in a convection oven at 105°C. The weight of the beakers was measured at equal time intervals, until it was constant. The last reading was recorded (B0).

Approximately 2 g of oven-dried soil (<2 mm) were weighed into the tared beakers to the nearest three decimal points (OS). Beakers were placed into the furnace and the temperature set to 450 °C. They were left overnight. When cool enough to handle, beakers were transferred to a desiccator and left to cool to room temperature and then weighed (FW).

Calculations

The ash percentage and the percentage of organic matter content by loss-on-ignition were calculated as follows:

$$\% \, Ash = \frac{FW - BO}{OS} \times 100$$ **equation 2.8**

$$\% \; Organic \;\; matter \; = 100 \; - \% \; ash$$ **equation 2.9**

2.2 Chemical extractions and analyses

2.2.1 Soil pore water extraction by centrifugation/filtration

Principle

The soil solution has been defined as the aqueous liquid phase of the soil and its solutes (SSSA, 1975). The solutes consist of dissolved electrolytes and gases (at equilibrium or quasi-equilibrium with definable solid and gas phases of the soil) and small quantities of other water-soluble compounds such as organic substances and metabolites. It is accepted that the soil solution has uniform macroscopic properties like electrolyte concentration and temperature, and that it can be isolated from the soil for its study in the laboratory (Sposito, 1989).

Isolation of the soil solution and its subsequent chemical characterisation have useful applications in studies of soil chemistry and biochemistry, plant nutrition, mineral stability, and assessment of environmental contaminants (such as toxic metals and pesticides) and their movement in soils (Soon, 1993).
At a microscopic scale the soil-water interface consists of a usually net-negatively charged particle surface surrounded by a diffuse layer of hydrated counter-ions, the micellar or inner solution. This inner solution merges into a homogeneous intermicellar or outer solution. It is generally accepted that it is the outer solution that constitutes the soil solution (Adams, 1974; Sposito, 1989). To date it is not

possible to quantify the chemical composition of the soil solution in situ, and several methods to attempt the isolation of the "true" soil solution have been devised.

Methods of studying the chemistry of soil solutions may be indirect (Beckett 1964; White and Beckett 1964) or direct. Direct methods require that the solution be isolated without changing its chemical composition. Several approaches have been used to this purpose:

- *Extraction of soil solution by compaction.* These methods are not satisfactory due to the interaction of double layers and salt sieving (Appello, 1977).

- *Isolation of soil by absorption on filter paper* (Hinkley and Patterson, 1973). These methods are not reliable because of the retention of ions and release of contaminants from filter materials.

- *In situ soil solution sampling, by suction into ceramic porous cup containers* (Wood 1973). These have often been used in hydrology and environmental monitoring, however, several studies show that sorption or contamination alters the chemical composition of the extracted solution (Hansen and Harris, 1975; Rauland-Rasmussen, 1989).

- *Column displacement.* Several variants of this method have been used since its introduction at the end of the 19th century (Nye and Tinker, 1977). It involves the use of a coloured solution to displace the soil solution. The reliability of this method has been demonstrated (Adams *et al.*, 1980). The disadvantages of the technique are the requirement for an

experienced operator, the long time involved and the need for large soil samples.

- *Centrifugal methods.* These are more often used as there is not a requirement for particular skills or previous experience. Small amounts (*e.g.* 10 g) can be used (Reynolds, 1984) and the soil solution can be extracted within 30-60 minutes. The recovery efficiency ranges from 3 to 30%.

To avoid chemical changes, it is recommended to use field moist soil whenever possible, and to carry out the extraction of the soil solution within 24 hours of sampling (Edmeades *et al.*, 1985). To ensure a sufficient amount of soil solution for analysis, it is possible to remoisten field-moist soil samples to a percentage of their WHC and allow them to re-equilibrate for at least 48 hours before extracting the soil solution (Soon, 1993).

Apparatus and materials

- Two-piece Perspex centrifuge tubes with plastic insert
- Polythene bags to fit in the Perspex body
- Filter paper (Whatman 40, 12.5 cm diameter)
- Two decimal place balance
- Centrifuge buckets and inserts
- Centrifuge Mistral 3000 (Sanyo Gallenkamp, UK)

Procedure

Two-piece Perspex centrifuge tubes (devised in-house) were used. A polythene bag was pushed through the top half of the Perspex body, with the opening in the bag overlapping the edges of the body. The

bag was rolled at the bottom to fit in the base and the two parts assembled, taking care that the bag was not caught in the joint. The plastic disk insert was placed in the bag and covered with the filter paper, fitting the sides of the bag.

Approximately 200 g of soil were weighed into the polythene bag, taking care that the soil stayed within the filter paper edge. The bag was closed and the two-piece Perspex body placed into a second polyethylene bag, to collect any leakage of soil solution. The Perspex body was placed into a centrifuge container. The procedure was repeated for another three samples, taking care that the final weights of the centrifuge containers were similar in order to balance them inside the centrifuge. The soil was centrifuged in the Mistral 3000 centrifuge for 1 hour at 3000 rpm, brake level 9, at 15 °C.

After centrifugation the Perspex base was removed and the solution collected into a bottle, by cutting a corner of the polythene bag. The solution obtained was weighed.

Problems associated with this method were the low percentage of soil solution recovery and the need for equilibration of samples with added water before centrifuging.

2.2.2 Microwave-assisted acid digestion for *pseudo*-total elemental analysis of soil

Principle

The trace element composition of a soil differs depending on pedogenic and anthropogenic inputs. Total analysis of elements

assists in determining the soil composition, as well as possible contamination.

Most trace element analytical techniques require the sample to be in solution form, which can be attained by several procedures, which in general should dissolve the sample completely (no insoluble residues), be reasonably quick and safe, have no possible sources of sample loss through volatilisation or adsorption onto the walls of the vessel; and avoid contamination from the reagents used in the dissolution process. The majority of dissolution procedures involve dry ashing or wet digestion using one or a combination of concentrated mineral acids.

Muffle ashing at 500-550 °C will decompose most organic matter, although problems can occur through volatilisation of As, Cr, Hg, Ni, Pb, Se and Sn. Wet digestion is often the preferred method for soils, sediments, biological tissues and blood (Ward, 1995).

Samples (geological and environmental) can be selectively attacked by treatment with standard mineral acids (hydrochloric, nitric or sulphuric acids, for example). *Aqua regia* (three parts HCl + one part HNO_3) is often used for the digestion of soil samples, although nitric acid alone and occasionally other acid mixtures, may be recommended for specialised applications. *Aqua regia* will leach most elements from soils with considerable efficiency, due to the complexing power of the chloride ion and to the catalytic effect of Cl_2 and NOCl and Cl^- present as the reaction takes place (Bock, 1979).

For some environmental samples, dissolution in cold dilute nitric acid may be a preferred preparation, for example, where the analysis is intended to determine the amount of analyte that is 'biologically available'. Increasing acid strength and temperature will release

increasing amounts of elements, even partially leaching many elements from silicate minerals, notably from clay minerals. A 'complete' analysis will, however, require the complete dissolution of the sample and for many environmental samples this will involve using hydrofluoric acid to break down any silicate minerals present (Walsh *et al.*, 1992). When using *aqua regia* digestion, the elemental concentrations found in the resultant solution are considered to be pseudo-total, as elements present in the silicate matrix are not fully released. An advantage of using *aqua regia* instead of hydrofluoric acid (which does digest silicates) is that volatile components such as As, Hg, Sb, Se, *etc.*, can be retained and that the total concentration of dissolved solids in the resulting filtered solution is minimised, this may be important for certain types of analysis.

Wet digestion using an open vessel is always subject to possible element volatilisation. Pressurised decomposition of the sample (0.1-0.2 g or 0.5-1.0 ml) with acid in Teflon® digestion bombs eliminates this problem and increases the digestion efficiency.

Microwave digestion provides both a closed system method and shorter digestion times. Optimal conditions depend on the sample (weight, composition, volume of digestion reagents, reaction temperature, pressure, and time) and the digestion system (especially power ratings).

Relevant available standard procedures: USEPA 3050 (open system digestion of sediments, sludges, soils and oils (USEPA, 1996)), USEPA 3051 (microwave assisted digestion of sediments, sludges, soils and oils (USEPA, 1998a)).

Method

This method was used for the determination of soil elemental compositions including the determination of total Cr. Variations in the digestion cycle parameters for plant material are indicated in the pertinent chapters.

Apparatus and materials

- Microwave digestion apparatus - CEM MARS5 (CEM, UK).
- Microwave digestion Teflon® vessels.
- Volumetric flasks (from 25 to 100 ml, depending on the material being digested)
- Filter paper - Whatman 542
- Graduated borosilicate pipettes, class A
- Borosilicate funnels to fit volumetric flasks
- Analytical balance
- Polythene tubes to store the diluted digests
- Reference material

Reagents[3]

- Hydrochloric acid (Conc.)
- Nitric acid (Conc.)
- Deionised water
- 2% (v/v) nitric acid solution

Procedure

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[3] Unless specified all acids and solvents used in this work were analytical reagent grade.

A method adapted from USEPA method 3051 (USEPA, 1998a) was used to digest the range of materials in the study.
Microwave vessels were washed with concentrated nitric acid using a special microwave cycle: Power = 1200 Watts, ramping time = 15 min., holding time = 15 min., maximum temperature = 200° C, pressure = 150 psig.

After the cycle finished, the vessels were left to cool for 5 min., taken out from the microwave digestion apparatus, placed in a fume cupboard and left to cool down to room temperature for about two hours.

The nitric acid in vessels was discarded and they were then rinsed thoroughly with deionised water at least three times and left to dry overnight.

Of the 14 vessels in each microwave digestion cycle, 12 contained samples, one a reference material and the other a reagent blank (*i.e.* no sample). Approximately 0.5 g of oven-dried soil was weighed into each digestion vessel and the weights recorded.

Deionised water (1 ml) was added to each vessel, then swirled gently, in order to wet the sample. Hydrochloric acid (9 ml) was added to each vessel, followed by 3 ml of nitric acid, and the vessel was swirled gently again to mix the soil and acid mixture. Vessels were immediately placed in the microwave digestion apparatus to maximise the microwave digestion of the soil and minimise *aqua regia* self-reaction.

Parameters for the microwave digestion apparatus were set up as follows: power = 1200 Watts, ramping time = 30 min., hold time = 20

min., maximum temperature = 200 °C, pressure = 175 psig. The cycle was then started.

After the cycle finished, the vessels were left to cool for 5 min., taken out from the microwave digestion apparatus, placed in a fume cupboard and left to cool down to room temperature for about two hours.

When the vessels were cool enough to handle, the pressure valves were carefully released and the vessels opened. The digest solutions were carefully filtered into the volumetric flasks. The digestion vessels were rinsed several times with a 2% (v/v) nitric acid solution into the filters. Finally, the filters were also washed several times and the solutions in the flasks were made up to the mark and mixed thoroughly.

Digests were stored in labelled polypropylene tubes and placed in the refrigerator until they were analysed by ICP-OES.

Possible problems associated with the method:
The microwave system has to be managed carefully to avoid explosions (fine powders have to be digested in small amounts and the digestion of materials with more than 20% fat content should be avoided), which can result in loss of sample and therefore a false result.

2.2.3 Alkaline digestion for extraction of hexavalent chromium

Principle

To quantify total Cr(VI) in a solid matrix, three criteria must be satisfied: (i) the extracting solution must dissolve all forms of Cr(VI),

(ii) the conditions of the extraction must not induce reduction of native Cr(VI) to Cr(III), and (iii) the method must not cause oxidation of native Cr(III) contained in the sample to Cr(VI) (USEPA, 1991).

It has been suggested that a heated carbonate-hydroxide solution is an effective extractant to operationally define total Cr(VI) in soils that contain native Cr(VI) or that present sufficiently high redox conditions to maintain Cr as Cr(VI) (James *et al.*, 1995). A modified method that uses Mg^{2+} (as magnesium chloride) in phosphate buffer minimises the potential reduction of Cr(VI) (USEPA, 1992a).

The alkaline digestion is used to extract hexavalent Cr(VI) from soluble, precipitated and sorbed Cr(VI) compounds in soils, sludges, sediments and similar waste materials. The digestion dissolves both water-insoluble (with the exception of barium chromate) and water-soluble Cr(VI).

The digests obtained can be analysed for Cr(VI) using a colorimetric technique (USEPA 7196a, described in Section 2.2.5) or spectrometric techniques.

Apparatus and materials

- Field moist soil samples
- Reference material
- 250 ml borosilicate beakers
- Watch glasses to cover the beakers
- Borosilicate measuring cylinders
- 250 ml conical flasks
- Ashless filter paper Whatman 542
- Thermometer

- Three decimal place balance
- Hot plate

Reagents

- Deionised water
- Carbonate-hydroxide solution (pH 11.8-12.3): 0.28 M Na_2CO_3 in 0.5 M NaOH

Procedure

Soil samples were field moist when extracted. Each sample was extracted in duplicate. The equivalent of 2.5 g ± 0.02 g of oven dried-soil material was weighed into 250 ml beakers. In addition, beakers containing a reference material and a blank (i.e. reagents only) were included. The carbonate-hydroxide solution (50 ml ± 0.1 ml) was added to each beaker. The beakers were placed on a preheated hot plate, covered with a watch glass to avoid evaporation and heated to a temperature of 85-95°C, which was maintained for 1 hour. The temperature was checked by means of a thermometer placed into a beaker containing the carbonate-hydroxide reagent solution. The beakers were swirled continuously.

Once cooled, the extracts were filtered into 100 ml volumetric flasks, rinsing the beakers and filters with deionised water. The solution in the flask was made up to the mark with deionised water. The digests were analysed by the 1,5-diphenylcarbazide colorimetric method (Section 2.2.5).

Possible problems associated with the method:

- The redox status of the soil may affect the extraction of Cr(VI).

- Extractions of soils containing high amounts of Mn may be biased due to possible reduction of Cr(VI) by Mn^{2+}.

Relevant standard method available: USEPA 3060a

2.2.4 Phosphate extraction for soluble and exchangeable Cr(VI)

Principle

Distilled water and phosphate buffer (pH 7.2) extractions have been used to operationally quantify and define soluble and exchangeable (adsorbed) forms of Cr(VI) in several soils under diverse redox conditions. Phosphate buffer (pH 7.2) has been suggested as a suitable extractant for adsorbed Cr(VI) due to the fact that the buffering effect at neutral pH should minimise reduction during extraction at a lower pH and avoid dispersion of organic matter at a higher pH. It also contains a competing anion concentrated enough to remove Cr(VI) from exchange sites without extracting reducing organic compounds (James and Bartlett, 1983).

The soluble and exchangeable fractions of Cr(VI) are useful parameters for estimating soil levels of Cr(VI) that may leach to groundwater or be uptake by plants and microorganisms (James and Bartlett 1984; James, 1994).

Bartlett and James (1977) suggested that a 10mM phosphate buffer at pH 7.2 was sufficient to extract Cr(VI) from environmental samples without extracting organic matter. In the samples used in this work Ca was found at concentrations several orders of magnitude greater than chromium. Calcium is known to form complexes with phosphate, in which case phosphate would complex

preferentially with Ca rather than exchange with CrO_4^{2-} sorbed on soil particles. To minimise the possibility of phosphate being used up completely for Ca complexation, in this work the concentration of phosphate was increased to 20 mM. Experiments showed that the increase in phosphate concentration yielded higher recoveries of Cr(VI), but also organic matter (measured at 254 nm). The concentration used was also constrained by the need neither to inhibit nor to stimulate *E. coli* pUCD607 (Section 3.1).

Apparatus

- 50 ml centrifuge tubes (Fisher Scientific, UK)
- Flask shaker (Stuart Scientific SF1)
- Graduated measuring cylinders
- Analytical balance
- Calibrated pH-meter Jenway 3305 (Jenway, UK)
- Centrifuge MSE Mistral 1000 (Sanyo Gallenkamp, UK)
- Filter paper - Whatman 542
- 50 ml conical flasks

Reagents

- Phosphate buffer (pH 7.2): prepared from equal volumes of 10 mM KH_2PO_4 and 10mM $KHPO_4$

Procedure

Extractions, including blanks, were carried out in duplicate. A soil sample of 1.0 g (± 0.01 g) was weighed into each 50 ml centrifuge tube, to which 40 ml of phosphate buffer were added. Tubes were shaken for 1 hour at 20°C and then centrifuged for 10 min at 4500

rpm. The supernatant was filtered into conical flasks and analysed immediately using the 1,5-diphenylcarbazide method (Section 2.2.5).

2.2.5 Spectrophotometric determination of Cr(VI)

Principle

Dissolved hexavalent Cr (0.5-50 mg/L), may be determined colorimetrically by reaction with 1,5-diphenylcarbazide in acid solution. A red-violet complex of unknown composition is produced. The reaction is very sensitive, the absorbance index per gram atom of chromium being about 40,000 at 540 nm (USEPA, 1992b).

The Cr(VI) reaction with 1,5-diphenylcarbazide is usually free from interferences. However, certain substances may interfere if the Cr(VI) concentration is relatively low. Hexavalent Mo and Hg salts also react to form colour with the reagent although the red-violet intensities produced are much lower than those for chromium at the specified pH. Concentrations of up to 200 mg/L of Mo and Hg can be tolerated. Vanadium interferes strongly when present in concentrations (10 times) higher than that of Cr(VI).

Iron in concentrations greater than 1 mg/L may produce a yellow colour, but the ferric iron colour is not detectable at 540 nm.

Apparatus and materials

- Spectrophotometer, for use at 540 nm, providing a light path of 1 cm or longer
- 25 ml volumetric flasks (Class A) with caps
- Glass or quartz cells

Reagents

- Deionised water
- Phosphoric acid (Conc.)
- Acetone
- 1,5-diphenylcarbazide
- Potassium dichromate stock solution - 100 mg Cr/L: 282.8 mg of dried potassium dichromate, $K_2Cr_2O_7$ (analytical reagent grade), in 1 litre of deionised water
- Cr(VI) solution for standard preparation 10 mg/L: 10 ml of potassium dichromate stock solution in 100 ml of deionised water
- Diphenylcarbazide reagent: mixture of solutions a and b (described below), stored for up to 15 days at 4 C in amber glass bottle.
    - Solution a: 380 mg of 1,5-diphenylcarbazide in 100 ml of acetone
    - Solution b: 120 ml of phosphoric acid in 280 ml H_2O

Procedure

Standards containing concentrations of 0.0, 0.04, 0.2, 0.4, 1.2 and 2.0 mg/L of Cr(VI) were prepared fresh at the same time and treated using the same procedure as for samples.

A 2.5 ml aliquot of the sample was transferred to 25 ml volumetric flasks and 2.5 ml of diphenylcarbazide reagent were added to the sample or standard, mixed by swirling gently and left to stand for 20 min. for the colour to develop. The solutions were then made up to the mark with deionised water.

Standards and sample analysis

The spectrophotometer settings were adjusted to read at 540 nm and the absorbance calibrated to 0 using deionised water. Absorbance cells were filled with the samples or standards and the absorbance measured. A standard check was used every 10 samples. Where required, an aliquot of the sample containing all reagents except diphenylcarbazide was prepared and used to correct the sample for turbidity (turbidity blank). A calibration curve was constructed by plotting absorbance values against mg/L of Cr(VI) and the Cr(VI) concentrations in samples were calculated.

2.2.6 Inductively Coupled Plasma-Optical Emission Spectrometry

Principle

In inductively coupled plasma - optical emission spectrometry (ICP-OES) the sample is introduced into a plasma (partially ionised gas) source that is both an atomisation and an excitation cell, where the sample is evaporated and dissociated into free atoms and ions (Boumans, 1987).

Most analytical plasma sources are electrical gas discharges at atmospheric pressure, usually in argon or in another inert gas, with high temperatures of normally more than 5000 K in the viewing zone. In ICP-OES, the plasma cell contains a quartz torch, consisting of three concentric quartz tubes through which the inert gas flows (coolant, auxiliary and carrier gases). Around the torch is a water-cooled, copper, 3-ring induction coil, which is connected to a radio-frequency generator, operating at 27.12 MHz and 1–2 kW. The plasma is formed by introducing electrons (Tesla discharge) into the

gas flowing past the coil. This initiates the plasma in the torch, which is subsequently sustained by the eddy-currents induced by the high frequency oscillating field around the coil (Boumans, 1987).

The physical characteristics of the ICP make it an ideal spectrometric source. Sample particles experience temperatures of up to 8000 K when passing through the plasma. When they enter the normal viewing zone (15—20 mm above the coil), the decomposition products of the sample have been delayed 2 μs in the plasma at 8000—5500 K. The delay-time and the temperature which particles experience in the ICP are about twice those of a dinitrogen oxide-acetylene flame. In contrast to various combustion flames, free atoms and ions are in a chemically inert atmosphere in the ICP, and this is the reason why their lifetime in the plasma is longer than in the flame (Walsh, 1992).

Inductively coupled plasmas are divided into two main groups: (i) high-power, nitrogen—argon ICP, and (ii) low or medium-power argon ICP. Both types of ICP are very stable and when analysing the same sample repeatedly the relative standard deviation is about 0.4%.

The sample solution is converted to a fine mist (aerosol) in the spray chamber by a nebuliser system and transported into the plasma by the carrier gas. The high temperature of the plasma, the dissociation of the analyte compound into atoms and ions and their excitation are as a result of collisions with other particles, mainly with free electrons. The excited state is unstable and the atom or atomic ion loses its excess energy either by collisions with other particles, or by a radiative transition to a lower energy level (spontaneous emission of radiation).

The wavelengths of the emission lines are characteristic of the elements present in the plasma source. The detection of radiation at particular wavelengths can be applied to the qualitative analysis of the sample and the intensities measured at these wavelengths to the quantitative analysis of the elements.

Potential interference effects in ICP-OES comprise nebulisation interferences, chemical interferences, ionisation interferences, and spectral interferences.

Nebulisation interferences are observed if the amount of the sample nebulised varies considerably as a function of time, which can be caused by alterations in the viscosity, in the surface tension, or in the solution density by matrix, salts or organic compounds and solvents. This may also occur to some extent for solutions with high mineral acid concentrations.

Difficulties may occur in the analysis of organic samples, such as oils or organic solvents, and samples with high salt concentrations, if the injector tube orifice clogs with carbon or salt deposit after prolonged spraying.

Chemical Interference caused by the formation of thermally stable compounds or radicals are unlikely, especially in ICP-OES. For example, the interferences caused by phosphate and aluminium in the determination of calcium are common in combustion flames, but not in conventional ICP. However, with low power ICP these effects might exist to some extent. In general, with increased plasma power, chemical interferences become smaller.

Ionisation Interference, where easily ionisable elements, such as alkali and alkaline earth elements, may alter the intensities of the

emission lines of the analyte, causes little or no change in the intensity of the spectral lines in ICP.

Spectral Interferences. Spectral interferences are important in ICP because emission lines that might be expected to be weak or nonexistent in other sources such as flames, arcs, or sparks, are quite intense. All spectral interferences originate from the inherent argon spectrum, or from line and continuum spectra of atomic and molecular species entrained or injected into the plasma. Spectral interferences may be classified into four principal groups: spectral line coincidence, overlap with nearby broadened line wing, spectral continuum, and spectrometer stray light.

Spectral line coincidence, or direct line coincidence, occurs when the monochromator is not capable of separating the analyte line from the matrix line. It is difficult to avoid spectral overlap interferences occurring at one of the fixed or preset lines, with sometimes the only possible solution being the separation of the analyte from the interfering matrix component by a suitable method. However, with instruments offering a wide selection of analyte wavelengths, these problems may be by-passed by moving to another wavelength that does not exhibit the interference (Lajunen, 1992).

A strong, broadened line of a matrix element in the vicinity of the analyte line may cause spectral interference by overlapping the analyte line (overlap with nearby broad line wing). This interference may be avoided by moving to another interference-free analyte line, by chemical separation, or by using background correction.

Spectral interference caused by one of the matrix components emitting a continuum spectrum at the analyte line wavelength (spectral continuum), may be overcome by changing the analyte line

or by using chemical separation. In some cases the interference caused by a background spectrum line which directly coincides with the desired analyte can be avoided by background correction.

The degree of interference varies from one different plasma source and spectrometer used to another. However, the most significant impediment to the effective use of any OES is spectral interference (Boumans, 1985).

Apparatus

The instrument used for ICP-OES analysis was a ThermoElemental IRIS multielement ICP (Winsford, UK). The operating conditions were as follows:

- Argon gas flows: Carrier, 0.7 L/min; Coolant 15 L/min, Auxiliary, 0.4 L/min.
- Power: 1.35 kW
- Sample introduction rate: 1 ml/min
- Integration time: 25 s per replicate (3 replicates per sample/standard)

Procedure

A set of fresh standards was prepared from metal ICP standard solutions (Spectrosol, Merck, UK), in concentrations from 0.1 mg/L to 100 mg/L. A blank was analysed for each set of samples and a standard check was inserted between every 10 samples in a run, in order to ensure that the sensitivity of the instrument was constant.

The following spectral lines (in nanometres) were chosen to calculate elemental concentrations: Al 396.1, B 249.6, Ca 317.9, Co 228.6, Cr

205.5, Cu 224.7, Fe 259.9, K 769.8, Mg 383.8, Mn 257.6, Na 588.9, P 213.6, S 182.0, Se 203.9, Ti 334.9, Zn 213.8.

Typical detection limits (mg/L, defined as 3 times the standard deviation, divided by the mean of 10 consecutive blank readings) were: Al 0.05, B 0.003, Ca 0.010, Co 0.005, Cr 0.005, Cu 0.015, Fe 0.003, K 0.5, Mg 0.05, Mn 0.001, Na 0.3, P 0.03, S 0.1, Se 0.01, Ti 0.01, Zn 0.003.

2.3 Biological analyses

2.3.1 Protocol for assessing the bioluminescence of *E. coli* pUCD607 using a rapid freeze-dried bioassay

Principle

This method used a strain of *E. coli* to which the *lux* gene had been inserted, the microorganism being grown in a suitable medium in vials and freeze dried. The protocol comprises three stages, resuscitation, assay and light production measurement.

Apparatus and Materials

- *E. coli* pUCD607 culture vial
- 0.1M KCl sterile (sterilised by filtration with 0.20 μm bacterial cellulose filters)
- 2 ml sterile needle and syringe

- 10 ml sterile universal bottle
- Vortex mixer
- Turbidimeter (Biolog, model 21906, USA)
- Luminometer Clinicon 2174-086 disposable measuring cuvettes polystyrene (Clinicon, Sweden)
- Luminometer (LKB 1251, Wallac, Germany)

Procedure

Resuscitation:

In order to resuscitate the culture, 2-3 ml 0.1M sterile KCl were injected into the vial containing the freeze-dried culture and mixed thoroughly on a vortex mixer.

The culture was then removed from the vial and transferred to a sterile universal bottle. The suspension was made up to l0 ml with 0.1 M KCl. The culture was incubated at 25°C and shaken at 150rpm for 30-45 min, until the turbidity measured 0.42 absorbance.

Assay:

The cuvettes were prepared while the culture was incubated, adding 0.9 ml of test solution to each labelled cuvette. Samples were analysed in triplicate, with each replicate analysed in a separate batch. The first cuvette of each batch contained a control with 0.9 ml of deionised water or matrix solution (i.e no analyte). When the culture was resuscitated, 0.1 ml of cell suspension was added to the first cuvette of batch 1 and mixed on the vortex mixer for ten seconds; after a total of 20 seconds, 0.1 ml of cell suspension was added to the next cuvette and the procedure repeated until batches were completed in order. After 15 min. exposure time, the bacterial light output was measured using the 1251 luminometer. Results

were calculated as percentages relative to the control. Materials used were autoclaved three times before washing or disposal.

2.3.2 Protocol for assessing the bioluminescence of *E. coli* pUCD607 using a fresh culture

Apparatus and materials

- Orbital incubator (Gallenkamp, UK)
- 250 ml conical flask
- 10 ml universal bottles
- Micro-centrifuge (MSE Micro centaur, Sanyo Gallenkamp, UK) and vials

Reagents and media

- Luria Bertani (LB) Broth (DIFCO media)
- 0.1M KCl
- kanamycin stock solution

Procedure

A single colony was selected from a plate *of E. coli* pUCD6O7, and used to inoculate l0 ml of LB broth containing 3Oμg/ml kanamycin in a 10 ml universal bottle, the culture being incubated overnight in the orbital shaker at 25°C and 150 rpm. One ml of culture was inoculated into a 250 ml conical flask containing 100 ml LB broth, and incubated as overnight. Cells were harvested after 16 hr incubation by transferring equal amounts of the culture into 1.5 m1 centrifuge vials and centrifuged. The supernatant was discarded and the cell pellet was resuspended with 1.5 ml 0.1M KCl and made up

to the mark. The cells were centrifuged twice, discarding the supernatant each time and resuspending the cell pellet to the 1.5 ml mark with 0.1 M KCl. The final cell suspension was mixed together by transferring the contents from the vials into a sterile universal bottle.

Bioassay:
Same procedure as with the freeze-dried protocol.

2.3.3 Phospholipid fatty acid extraction

Adapted from Frostegård *et al.* (1993):

Principle

The signature lipid biomarker (SLB) analysis provides quantitative insight into important attributes of microbial communities: viable biomass, community structure and nutritional status.

The phospholipid ester-linked fatty acids (PLFAs) are taxonomically relevant constituents of the cell membranes of all organisms. When determining the total PLFAs in a system, it is possible to obtain information on the potentially viable biomass. Viable microorganisms have an intact membrane containing phospholipids (and PLFAs) that are hydrolysed by cellular enzymes within minutes to hours of cell death (White *et al.*, 1979), rendering a lipid diglycerid (DG) form and a free phosphate group. As the resulting DG contains the same signature fatty acid as the phospholipids, it is possible to contrast viable to non-viable microorganisms when comparing PLFAs with DGs (White, 1995).

The analysis of SLB also provides a quantitative definition of the microbial community structure. Specific groups of microbes often contain unusual lipids (White *et al.*, 1978; Tunlid and White, 1991). Patterns of the prominent PLFAs from isolated microbes after growth on standardised media are used to differentiate over 8000 species with the microbial identification system (MIDI, Newark, DE).

The method used in this work was based in the Bligh and Dyer fatty acid extraction method [Bligh and Dyer, 1959). It used a mixture of chloroform, methanol and citrate buffer (1:2:0.8) to extract lipids from the soil (the amount of soil extracted depended on the organic matter content) that are subsequently separated by column chromatography into neutral-, glyco- and phospholipids. The phospholpids are then converted into their methyl esters through mild methanolysis (fatty acids methyl esters, FAMES) and separated by gas chromatography (Frostegård and Baåth, 1996). The PLFAs were calculated with 17:0 methyl ester as the internal standard.

Apparatus and materials

- 10 ml glass tubes with Teflon-lined screw caps
- Vortex mixer
- Centrifuge mistral 2000
- Scintillation vials
- Solid phase extraction silicic acid columns with a sorbent mass of 500 mg and reservoir volume of 6 ml (IST silica International Sorbent Technology)

Reagents and standards

Reagents and standards

- 'Bligh and Dyer' extractant - chloroform: methanol: citrate buffer (1:2:0.8), prepared fresh every week.
- 0.15 M citrate buffer - 31.52 g of citric acid dissolved in 1 L of deionised water and adjusted to pH 4.0 with 2M NaOH. Prepared weekly.
- Acetone
- Hexane: chloroform (4:1 v/v)
- Methanol: Toluene (1:1 v/v)
- 1 M acetic acid
- Internal Standard - 6 mg of 17:0 methyl ester dissolved in 250 ml methanol (the solution was stored at a temperature between 3 and 5 °C)
- 0.2 M KOH (prepared when used)

Procedure

Extraction:

From 0.5 to 2.0 g were extracted with 1.5 ml citrate buffer, 1.9 ml chloroform, 3.75 ml methanol and 2 ml 'Bligh and Dyer' mixture. The supernatant was collected and 3.1 ml choloroform and 3.1 ml of citrate buffer were added in order to separate phases.

The aqueous layer was removed and discarded. The organic phase was transferred to a scintillation vial. The vials were placed under nitrogen and the solvent evaporated at 40°C. One ml methanol was added, the vials placed under nitrogen and the solvent evaporated to dryness. A further 1 ml methanol was added and vials evaporated to dryness under nitrogen. The vials were stored at -20°C.

Lipid Fractionation:

The silicic acid column was washed with 5 ml chloroform. The sample was dissolved in chloroform and placed onto the top of the column. Neutral lipids were eluted with 5 ml of chloroform and eluate collected in a scintillation vial. Glycolipids were eluted with 5 ml acetone and collected into a scintillation vial. Phospholipids were eluted with 10 ml of methanol and collected in a separate scintillation vial and then transferred to a glass culture tube for evaporation to dryness at 40°C under nitrogen. After evaporation 200 µl of the internal standard were added to the vial and evaporated to dryness under the same conditions. Vials were stored at –20°C.

Mild alkaline methanolysis:

The sample was re-dissolved in a mixture of methanol and toluene (1:1 v/v) and 1 ml 0.2M KOH added, before incubation in a water bath for 15 min at 37°C. After incubation 2 ml hexane:chloroform (4:1), 0.3 ml 1 M acetic acid and 2 ml deionised water were added and then mixed in a vortex mixer and centrifuged for 10 min at 1500 rpm. The organic phase was transferred to the same scintillation vial as the first organic phase. The solvent was evaporated at 40°C under nitrogen. The dried sample was stored until required for analysis, when it was transferred to a GC vial (containing a glass insert) using 3 x 150 µL isohexane (in 0.0001 % butylated hydroxytoluene).

The solvent was evaporated to approx 100 µL at 40°C under nitrogen and the vial sealed. The resulting fatty acid methyl esters were separated on a gas chromatographer.

2.3.4 Dye test for carbon utilisation measurement in microbial communities

Principle

The degradation of organic matter is a property of heterotrophic organisms and it is commonly used to indicate the level of the microbial activity in several environments, including soils. Diverse techniques have been developed to measure decomposition rates, including the measurement of CO_2 evolution or O_2 uptake, and the determination of the decrease in organic matter. Either the release or uptake of CO_2 or O_2 has generally been used to determine the microbial activity in soils.

Various methods are available for detecting changes in CO_2 concentrations, including gravimetric and titrimetric analyses for CO_2 trapped in aqueous alkali solutions; and manometric, gas chromatographic, infrared, mass spectrometric, and gas spectroscopic methods (Anderson, 1982).

Both basal respiration, which derives from the available organic material in soil, and substrate induced respiration (SIR, respiration induced by the addition of easily degradable organic substrates) can be determined in soil under a variety of experimental conditions. The dye test method is being developed at the Macaulay Land Use Research Institute (Patent applied for: UK Patent No. 0118620.4) and it uses a colorimetric analysis to detect CO_2 production, as proposed by Rowell *et al.* (1995).

Apparatus and materials

- 2 µl, deep multi-well plates (96 wells)
- 300 µl multi-well plates (96 wells)
- Muslin sachets containing soda lime
- 100 ml volumetric flasks
- 250 ml conical flasks
- Autoclave
- Vmax multi-well plater reader with Softmax software

Reagents

- Carbon sources (see individual set up)
- Deionised water
- Soda lime
- Basal respiration dye mixture: Cresol red (1.5 mg), potassium chloride (1.118 g), sodium bicarbonate (0.0126 g), deionised water (100 ml)
- Substrate induced respiration dye mixture formula: Cresol red (1.5 mg), KCl (1.118 g), $NaHCO_3$ (0.0252 g), deionised water (100 ml)
- Noble agar (1.5 g in 50 ml of deionised water)

Procedure

Soil conditioning:

A subsample of 50 g soil was sieved through a 2 mm mesh and placed into a 28 x 20 cm polythene bag. If necessary the soil moisture content was adjusted to 40 % water holding capacity (which depended on each soil type). Soils were incubated for 48

hours at 25°C inside a conditioning cabinet containing beakers with soda lime and deionised water.

Carbon source and dye plate preparation:
Carbon source stock solutions were prepared in advance and stored at 4°C until used, normally within 1 week. The concentrations of individual carbon solutions were such that the final concentration of carbon added to soils was 30 mg C per g water in the soil.

Basal respiration and substrate induced respiration dye test plates were prepared in advance. Cresol red, KCl and $NaHCO_3$ were dissolved in 100 ml deionised water. The dye mixture was incorporated in noble agar and plates were filled. The plates were left to solidify, covered with seals and stored in the dark inside polythene bags containing a sachet of muslin filled with soda lime. The plates were normally used within 12 to 48 hours of preparation.

Soil incubation:
Approximately 10 g of conditioned soil were used for each deep well plates, allowing 6 well columns per soil sample, *i.e.* two soils (approximately 5 g of each) were tested per deep well plate.

To test the soil basal respiration, the absorbance of a dye plate with 0.5% $NaHCO_3$ was measured (T_0). The dye plate was inverted and immediately placed over a deep well plate containing the soil, and sealed. The procedure was repeated for each deep well plate. The plates were incubated for 6 hours at 25 °C and the absorbance of the dye plate read and recorded (T_6).

To test the substrate-induced respiration, deionised water and 15 carbon sources were added in triplicate to each soil, according to the plate design. The absorbance of a plate with 1% NaHCO3 was read,

the plate was placed over the deep well plate, sealed and incubated for further 6 hours, after which the absorbance of the dye plate was read. The absorbance obtained for both basal and substrate-induced respiration was converted into mg/L CO_2 using a CO_2 calibration curve (Campbell *et al.*, 2001).

2.3.5 Measurement of inhibition of root growth

(Adapted from ISO 112669-1:1993 (E))

Hordeum vulgare (Barley) seeds were germinated in darkness at room temperature, distributed on a bed of paper towels moistened with distilled water. Once the radicles had emerged and reached 2 mm in length, they were planted in germinating pots containing 200 g of soil. The amount of water needed to achieve 70% WHC was added (calculation described in Annex 2).

Pots were placed in controlled environment cabinet (Control Environments LTS (CONVIRON®), Winnipeg, Canada) under the following conditions:

- Day length: 14 hours, including 1 hour to increase and decrease the light intensity (sunrise and sunset)

- Initial temperature: 20°C during the day and 17°C during the night with a ramping of 1.5 hours at the beginning and the end of the day light period.

- Relative humidity: 70%

- Light input: light was provided using a combination of equal amounts of a metal halide and a high-pressure sodium lamp,

providing a photosynthetic radiation of 500 - 550 $mm^{-2}s^{-1}$ at plant level.

The growth time used was five days, after which plants were harvested and the length of the roots measured.

2.4 Statistical methods

A series of statistical methods were used to analyse the data obtained (Miller and Miller, 2000):

- *Standard statistics (mean, standard deviation, standard error)*: calculated in order to define data sets.

- *Regression analysis*: used to obtain Cr concentration-response curves with the range of organisms or biological parameters tested.

- *Correlation matrices*: used to find possible relationships between chemical and biological variables.

- *Analysis of variance*: used to identify the effect of different treatments on the biological parameters measured.

- *Principal components analysis*: used to separate groups and reduce variables on PLFA analysis (Fritze *et al.*, 2000).

Chapter 3

Chromium (VI) Toxicity to a Bioluminescent Construct (pUCD607) of *Escherichia coli*

3.1 Introduction

As discussed previously, chemical analyses cannot by themselves solve the problems associated with the distinction of "real" risks posed by chromium (or any other toxicant) in environmental samples. Bioassays study the response of an organism(s) to contaminant(s). These involve many different biological mechanisms, both natural and genetically induced. Response can be assessed at

any level of biological organisation through the use of single species, a battery of single species or communities exposed to the same analyte (Luoma, 1995).

Single species bioassays test sub-lethal stress or survival in adults or in individuals from a sub-adult life stage. These are widely used in environmental toxicology, perhaps because they balance the advantages and disadvantages of other approaches. They are relatively easy to manage, control and standardise. Standardised methodologies allow reliable and repeatable results and relatively rapid data collection (Maltby and Calow, 1990). Luminescent biosensors have been used in the past to determine the toxicity of metals including Zn, Cd, Hg and Cr (Campbell *et al.*, 2000; Corbisier *et al.*, 1996; Ribo *et al.*, 1989; Garcia *et al.*, 1994).

This chapters reports on the study of Cr(VI) (in synthetic solutions) toxicity to the bioluminescent bacteria *E. coli* pUCD607

3.2 Objectives and hypothesis

- To determine conditions under which bioassays could be performed in order to investigate Cr(VI) toxicity;

- to study, over a range of pHs, the toxicity of Cr(VI) to *E. coli* pUCD607 in synthetic solutions;

- to obtain Cr(VI) dose-response curves at different pHs and calculate EC_{50} values which could serve as a basis to compare with the toxicity of Cr-containing environmental samples;

- to study the relationship between Cr(VI) speciation and toxicity

The principal hypothesis being tested in this chapter is that the toxicity of Cr(VI) varies with pH and that chemical, as well as biological, factors are responsible for the observed toxicity.

3.3 Preliminary experiments to determine conditions for the bioassays

In order to determine the conditions to carry out the bioassays to investigate the toxicity of Cr(VI), a series of preliminary experiments was carried out. Slight modifications to the original experimental procedure to use *E. coli* pUCD607 (Bundy *et al.*, 1999) were proposed, such as standardisation of resuscitation and exposure time, and the use of phosphate buffer as solution matrix. They are discussed in the following sections.

3.3.1 Standardisation of resuscitation time

Previous research using freeze-dried cultures of *E. coli* pUCD607 suggested a resuscitation time of 30 min[1]. Although the first batch of bioassays in this work was carried out using the suggested time, this resulted in very low luminescence and high variation among replicate samples (data not shown). In order to further investigate the optimum time for resuscitation of freeze-dried cultures, the light output of a single culture was measured at different times. A freeze-dried culture was suspended in 2 ml of sterile 0.1M KCl and transferred to a universal bottle with a total volume of 10 ml of 0.1M KCl. The culture was incubated at 25°C and light output was

[1] Resuscitation time is defined in Section 2.3.1.

measured in a Luminometer (LKB 1251, Wallac, Germany) after 30, 45, 55 and 60 minutes.

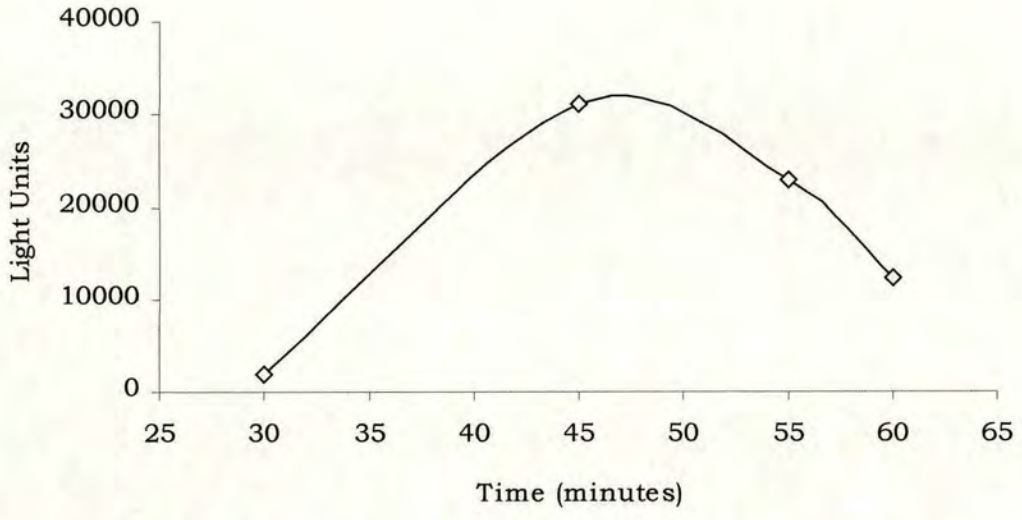


Fig. 3.1 Luminescence of an *E. coli* pUCD607 culture measured at different incubation times during the resuscitation stage.

It was found that 45 minutes resulted in the highest production of light (Figure 3.1) and that after 50 minutes of incubation, light output started to decrease.

As the detection of light produced by luminescent bacteria is a function of the population size (Meighen, 1991) it was decided that it would be more convenient to standardise the resuscitation period to the turbidity of the sample. The turbidity of three different cultures of *E. coli* pUCD607 in 10 ml of 0.1M KCl incubated at 45 minutes was measured. The absorbance recorded was 0.42, which was used in all bioassays with *E. coli* pUCD607.

After some months it was noted that the absorbance of 0.42 was not reached in 45 minutes and that more time was required to achieve the same turbidity. Therefore incubation times varied, but the absorbance was kept the same in all experiments.

It may be possible that the differences in the incubation time required for reaching the desired absorbance, were due to effects of time on the freeze dried culture. Some cultures were stored up to a year. This suggests that even if the culture is kept freeze dried, the ability of cells to recover and reproduce might be impaired after a long storage period.

3.3.2 Exposure time of bacteria to analyte

Exposure time and exposure concentration are related. The shorter the exposure time, the higher the concentrations needed to elicit a response; test species respond to progressively lower concentrations as exposure time increases (Luoma, 1995). The exposure time used in a bioassay will depend on the test organism and the toxicity of the analyte. An exposure time where toxicity results were not unduly influenced by the natural variation of light was sought. Campbell *et al.* (2000) reported the use of 20 minutes as exposure time when investigating the toxicity of Cd and Zn to *E. coli* pUCD607. Villaescusa *et al.* (1997) used 15 min. exposure time to investigate the toxicity of Cr to the Microtox® biosensor. In order to avoid the effect of natural light depletion and to obtain results that could be compared to those available for Cr toxicity in the literature, a 15 min. exposure time was selected for these experiments.

3.3.3 Effect of using phosphate buffer as bioassay matrix

As mentioned previously, the route of exposure in a bioassay may also affect the results (Luoma, 1995). The most common approach is to test the toxicity of samples in solution, in a synthetic solution prepared in the laboratory including buffers and mixtures with organic solvents (Campbell *et al.*, 2000; Bundy *et al.*, 1999), or environmental samples such as sea or lake water, soil or sediment pore-waters (Forge *et al.*, 1993; Carr *et al.*, 1989; Chaudri *et al.*, 2000).

As a solution of phosphate buffer was to be used to extract soluble and exchangeable Cr from environmental samples, it was decided that all bioassays in this work would be carried out in a matrix of 0.02M phosphate, instead of a 0.2M KCl solution, used in the original protocol (Campbell et al., 2000). Therefore, it was necessary to study the effect of phosphate on the bacterial luminescence. The initial hypothesis was that at the concentration of phosphate used, there would not be a significant effect on bacterial luminescence, compared with deionised water or a 0.1M KCl solution.

Solutions of different phosphate concentration were prepared using KH_2PO_4 and adjusted with 0.1M NaOH to pH 7.0. Solutions were assayed as described in Section 2.3.1.

Results from the assay using deionised water as control (Figure 3.2) suggested that the light output of bacteria was not significantly different at a concentration of 0.02M phosphate than that of water at the same pH. The highest production of light was found at a concentration of 0.5M phosphate. Luminescence was significantly reduced above 1.5M phosphate.

Using 0.1M KCl as control (Figure 3.3) produced more light compared to 0.02M phosphate and was equivalent to the luminescence from the 0.4M phosphate.

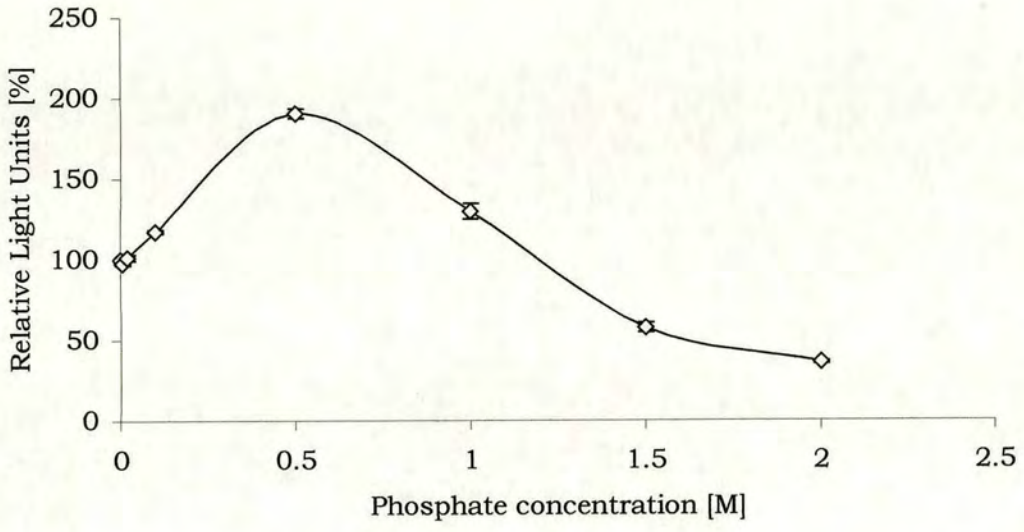


Fig. 3.2 The effect of phosphate concentration on *E.coli* pUCD607 at pH 7.0, compared with a deionised water control.

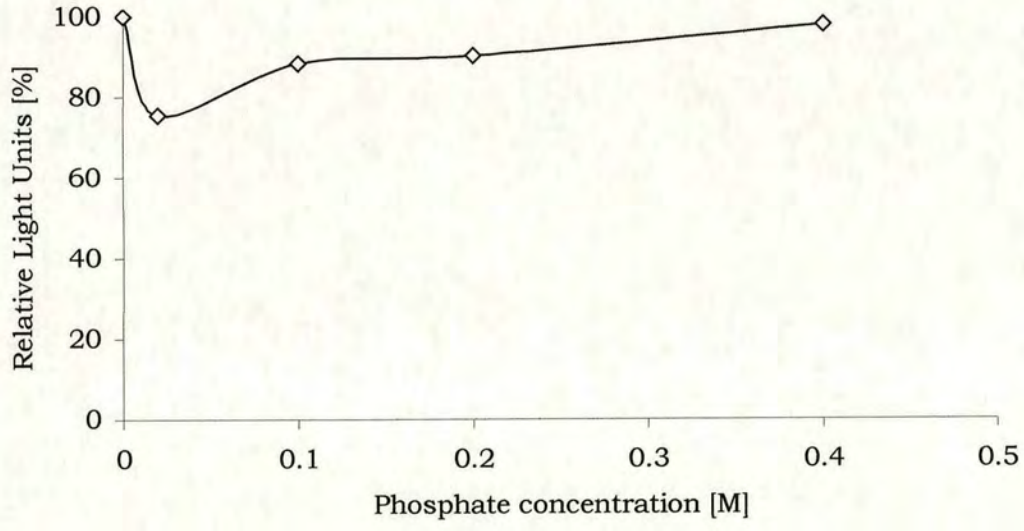


Fig. 3.3 The effect of phosphate concentration on *E. coli* pUCD607 at pH 7.0, compared with a 0.1M KCl control (no phosphate).

Potassium chloride is typically used to regulate osmotic pressure in bacterial media. In this case, phosphate might not provide as good conditions for the bacteria to perform their vital functions. However, comparison with water showed the effect might not be pronounced and a concentration of 0.02M phosphate could be used as the matrix for the bioassays, even though the optimal concentration would have been 0.5M phosphate.

James *et al.* (1997) stated that 0.02M phosphate is an optimal concentration for the extraction of exchangeable and soluble Cr(VI) and that concentrations above that would also extract organic matter, which would interfere with the chromium colorimetric analysis (Section 2.2.5).

3.3.4 Effect of pH

There is an optimum pH for bacteria to perform their vital functions. To investigate the pH range in which experiments could be carried out without significantly disturbing the functionality of *E. coli* pUC607, a series of 0.02M phosphate solutions adjusted to pH in the range 5.5 to 10 was carried out. Solutions were prepared using KH_2PO_4 and 0.1M NaOH with a concentration of 0.02M phosphate.

The hypothesis tested was that there was an optimum pH range in which the bacteria would produce the greatest amount of light, but below and above this optimum, light production would be depleted.

The bioassay was carried out as described in Section 2.3.1. The light output was measured after 15 minutes exposure.

In order to calculate the percentage relative light units (%RLU), the optimum pH at which the highest production of light was recorded was used as the 100 percent benchmark. The highest amount of light was found at pH 7.0 (Figure 3.4). Over 80% RLU was observed between pH 6.5 to 8.5, with the maximum at pH 7.0. At pH 5.5 RLU had depleted to below 50%. At pH 10, luminescence was very low and this can be considered as highly toxic for the bacteria. We might conclude that the same would be found when the bacteria are in contact with solutions at pH values below 4.0.

This experiment showed clearly that the pH of the matrix can be a major determinant of bacterial luminescence and confirms the hypothesis of an optimum pH range, which could be considered between 6.5 and 8.5.

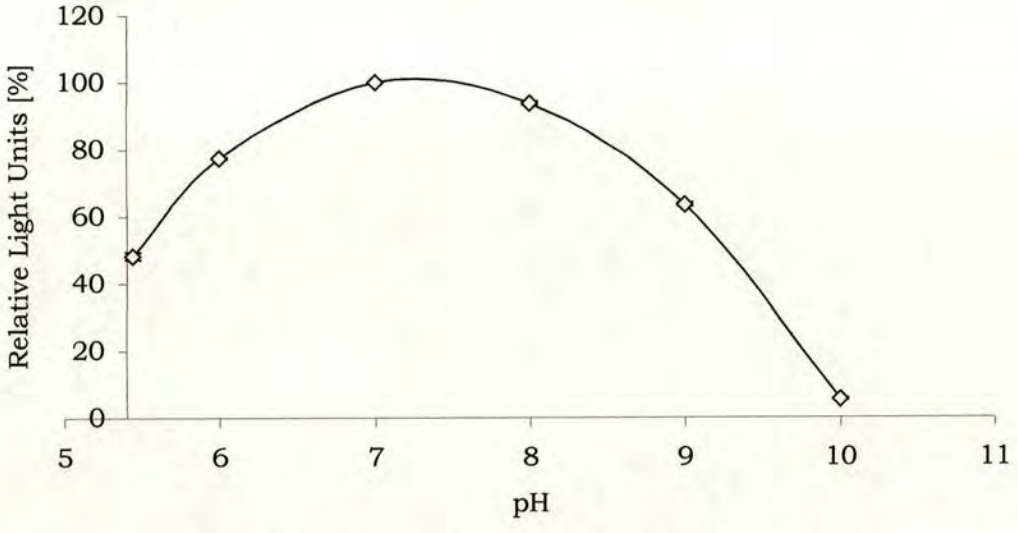


Figure 3.4 The effect of pH on the luminescence of *E. coli* pUCD607

From this series of experiments it was concluded that:

- the resuscitation period should be standardised to the time required to reach and $A_{(410)}$= 0.42;

- the resuscitation time varies with and within batches of cultures, probably due to storage time;

- the exposure time should be set at 15 minutes;

- it was possible to use a 0.02M phosphate solution as matrix for the bioassays as it would not affect significantly the production of light and could be used as a reference solution;

- *E. coli* pUCD607 can perform vital functions adequately between pH 6.5 and 8.5 without excessive stress. Outside this pH range, there is a deleterious effect on the luminescence of bacteria which constitutes an independent source of variation. This must be considered when comparing samples with different pH.

3.4 Chromium (VI) effect on the luminescence of *E. coli* pUCD607 at different pH

The response of *E. coli* pUCD607 to Cr(VI) in solution was investigated at different pH (4.5 to 9.0) using a series of Cr(VI) concentrations at each pH.

In order to prepare the solutions, increasing volumes of a Cr(VI) stock solution (from $K_2Cr_2O_7$) were added to a phosphate matrix (KH_2PO_4); the pH was adjusted to 5.8, 7.0, 8.0 or 9.0 (±0.1) with 0.1M NaOH and the total volume made up to 25 ml. The phosphate concentration was kept constant at 0.02M. Solutions were also

prepared without adjusting the pH. The pH of these solutions was 4.4 (±0.1).

Solutions at a given pH were assayed (in triplicate) and compared with a 0.02M phosphate control solution with no Cr(VI), at the same pH. When working with living microorganisms different factors can contribute to variations, culture, time of storage, ability of the microorganisms to recover and grow, *etc.* If bioassays are going to be used to compare different samples and conditions, reproducibility of results is important; for that reason, at least two independent experiments were carried out at each pH, using different freeze-dried vials and solutions from different batches. The %RLU were calculated using the control as the 100% benchmark. The relative light units were plotted versus the range of Cr(VI) concentrations used in each bioassay, in order to construct a Cr(VI) concentration-response curve.

The statistics computer package, Genstat 5.3 (NAG Ltd, Oxford, UK), was used to perform a regression analysis on the results of the two experiments at each pH. The %RLU was the variable modelled; the replicates for each experiment were included; Cr was considered as the treatment structure and experiments were blocked. An exponential or asymptotic regression curve (Equation 3.1) was found to be the curve which best represented the experimental values. The addition of more parameters, resulting in the use of other curves (*i.e.* double exponential, critical exponential, exponential with linear trend) was not justified by the small change in the degree to which other models explained the data variance (Miller and Miller, 2000).

The accumulated analysis of variance was used to determine whether there was a significant difference in bacterial response between experiments.

The parameters obtained from the regression analysis were incorporated into the exponential equation and used to calculate effect concentration (EC) values. The exponential equation,

$$y = A + BR^x \qquad \textbf{equation 3.1}$$

was solved for x in order to find EC values,

$$x = \frac{\ln \frac{y-a}{b}}{\ln r} \qquad \textbf{equation 3.2}$$

where y corresponds to the EC level being calculated, i.e. 25, 50, 75.

3.4.1 Bacterial response to Cr(VI) at pH 4.5

Seven different concentrations of Cr(VI) were tested in experiment 1, but the results showed a big gap in responses between 0 and 50 mg/L Cr(VI), therefore additional concentrations in that range were included in experiment 2.

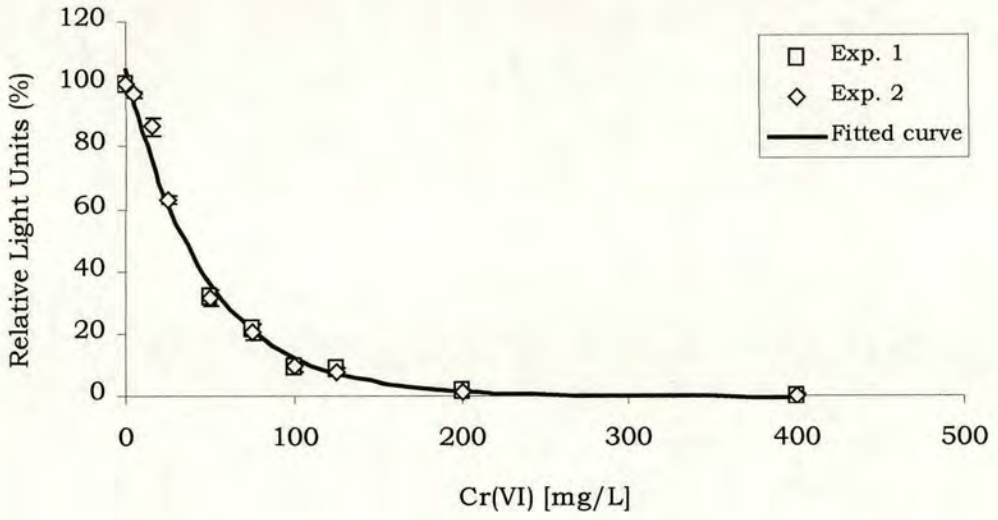


Fig. 3.5 Concentration-response curves for the effect of Cr(VI) on the luminescence of *E. coli* pUCD607 at pH 4.5 - Data for two independent experiments, with cultures from two different vials. In the case of experimental data, the mean of three replicates is plotted. The continuous line corresponds to the fitted values for the two experiments.

A highly significant ($P<0.001$) negative exponential response was found, indicating that Cr(VI) was toxic to the bacteria (Figure 3.5). The repeatability within experiments (mean SE=1.38) and the reproducibility of experiments (mean SE=0.38) may be considered good.

The parameters obtained for the fitted exponential curve in Figure 3.5 were A=-0.45, B=204.73 and R=0.97901. Using these parameters, the following EC values were obtained (all in mg Cr/L solution): EC_{25}= 15, EC_{50}=34, EC_{75}=67, EC_{100}=257, indicating that beyond 257 mg/L of Cr there is no more production of light.

The accumulated analysis of variance obtained from regression analysis indicated that there was not a significant difference in bacterial response between experiments (P=0.246).

3.4.2 Bacterial response to Cr(VI) at pH 5.8

Chromium (VI) was found to be toxic to bacteria at pH 5.8 (Figure 3.6), a highly significant negative exponential response ($P<0.001$) was observed.

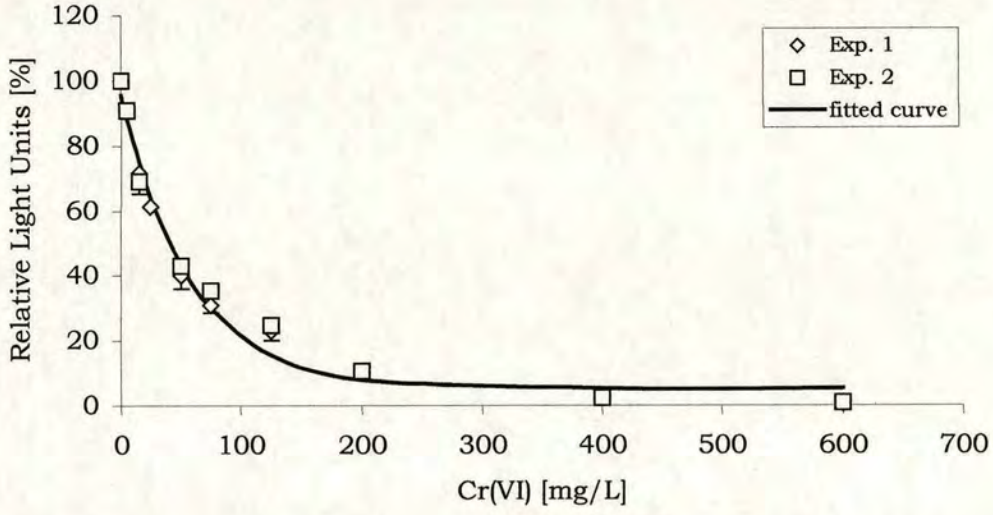


Fig. 3.6 Concentration-response curves for the effect of Cr(VI) on the luminescence of *E. coli* pUCD607 at pH 5.8 - Data for two independent experiments, with cultures from two different vials. In the case of experimental data, the mean of three replicates is plotted. The continuous line corresponds to the fitted values for the two experiments.

The repeatability within experiments (mean SE=1.42) and the reproducibility between experiments (mean SE=0.68) may be considered good, although the variation of data was greater than at pH 4.5.

The parameters of the fitted curve in Figure 3.6 were A=5.21, B=90.21 and R=0.9829. From this equation the following EC values were obtained (all in mg Cr(VI)/L solution): EC_{25}=15, EC_{50}=41 and EC_{75}=88. It was not possible to calculate an EC_{100} as according to the model, the curve would not fall to 0% light production, although

experimental data did reach values as low as 1%. In this case the fitted values at high Cr(VI) concentrations are slightly higher than those observed experimentally.

The accumulated analysis of variance from the regression analysis showed that there was not significant difference in bacterial response between experiments (P=0.844).

3.4.3 Bacterial response to Cr(VI) at pH 7.0

Figure 3.7 shows the concentration-response curves for experiments at pH 7.0. Again, Cr(VI) was toxic to bacteria, inducing a highly significant negative exponential response (P<0.001). The shape of the fitted curve is different from those obtained at lower pH values. The %RLU for both fitted and experimental values did not reach values below 10; the curves showed that for Cr(VI) concentrations above 1000 mg/L the change in luminescence is very low (asymptotic behaviour). The repeatability within experiments (mean SE=0.85) and the reproducibility between experiments (mean SE=0.46) may be considered good.

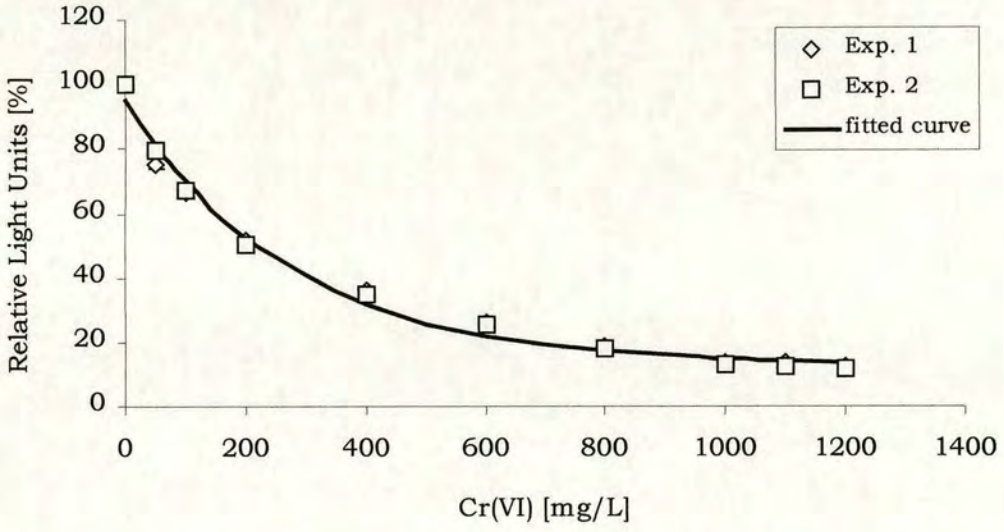


Fig. 3.7 Concentration-response curves for the effect of Cr(VI) on the luminescence of *E. coli* pUCD607 at pH 7.0. Data for two independent experiments, with cultures from two different vials. In the case of experimental data, the mean of three replicates is plotted. The continuous line corresponds to the fitted values for the two experiments.

The parameters of the fitted curve in Figure 3.7 were A=12.824, B=82.3 and R=0.996359. From this equation the following EC values were obtained (all in mg Cr(VI)/L solution): EC_{25}=77, EC_{50}=218 and EC_{75}=524. It was not possible to calculate an EC_{100} as according to the model, the curve would not fall below 13% RLU. This model fitted experimental %RLU values better than the models for lower pH.

The accumulated analysis of variance from the regression analysis showed that there was not a significant difference in bacterial response between experiments (P=0.865).

3.4.4 Bacterial response to Cr(VI) at pH 8.0

Figure 3.8 shows the concentration-response curves for experiments at pH 8.0. The highly significant negative exponential response ($P<0.001$) showed that Cr(VI) was toxic to bacteria. The shape of the fitted curve was again different from those at lower pHs and the experimental %RLU values showed that above 1000 mg/L Cr(VI), increasing concentrations of Cr(VI) did not have a significantly different effect on bacterial luminescence, *e.g.* the solution containing 1600 mg/L Cr(VI) changed the %RLU by just 1 unit. This again indicated an asymptotic behaviour.

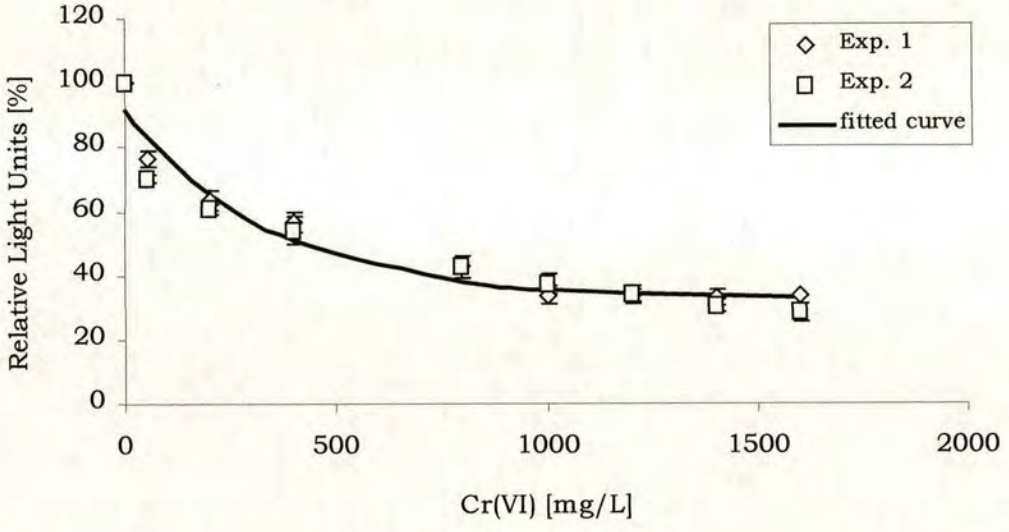


Fig. 3.8 Concentration-response curves for the effect of Cr(VI) on the luminescence of *E. coli* pUCD607 at pH 8.0. Data for two independent experiments, with cultures from two different vials. In the case of experimental data, the mean of three replicates is plotted. The continuous line corresponds to the fitted values for the two experiments.

In this set of experiments repeatability within experiments (mean SE=2.39) and reproducibility between experiments (mean SE=1.27) may be considered good.

The parameters of the fitted curve in Figure 3.8 were A=32.33, B=58.94 and R=0.997117. The following EC values were obtained from the concentration-response curve (all in mg Cr(VI)/L solution): EC_{25}=95, EC_{50}=417. It was not possible to calculate the EC_{75} and EC_{100} values. According to the model, the curve would not fall below 33% RLU, which agreed with the experimental data.

The accumulated analysis of variance from the regression analysis indicated that there was not significant difference in bacterial response between experiments (P=0.436).

3.4.5 Bacterial response to Cr(VI) at pH 9.0

Figure 3.9 shows the concentration-response curves for experiments at pH 9.0. Again, a highly significant (P<0.001) negative exponential response indicated Cr(VI) was toxic to bacteria. The shape of the curves were similar to those obtained at pH 8.0. Experimental and modelled data showed that above 1000 mg/L Cr(VI), increasing concentrations of Cr(VI) did not have a significantly different effect on bacterial luminescence; at a concentration of 1600 mg/L Cr(VI), the %RLU values changed by approximately 5 units. This again indicated an asymptotic behaviour.

In this set of experiments repeatability within experiments (mean SE=1.35) and reproducibility between experiments (mean SE=1.06) may be considered good.

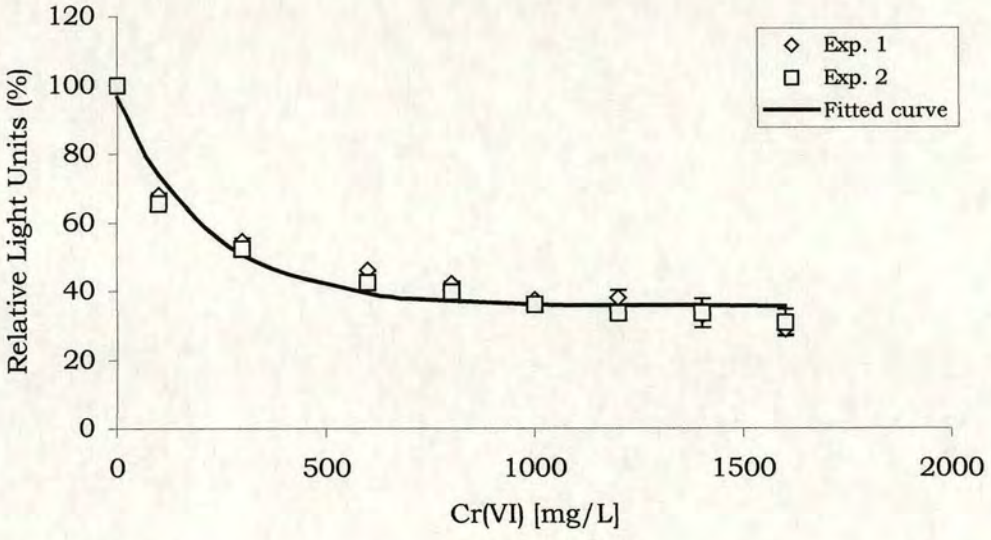


Fig. 3.9 Concentration-response curves for the effect of Cr(VI) on the luminescence of *E. coli* pUCD607 at pH 9.0. Data for two independent experiments, with cultures from two different vials. In the case of experimental data, the mean of three replicates is plotted. The continuous line corresponds to the fitted values for the two experiments.

The parameters of the fitted curve in Figure 3.9 were A=35.6, B=60.93 and R=0.995372. The following EC values were obtained from the concentration-response curve (all in mg Cr/L solution): EC_{25}=94, EC_{50}=311. EC_{75} and EC_{100} could not be obtained from the equation, as the model did not reach below 35% RLU. Experimental values showed a similar behaviour. A decrease in the EC_{50} value at pH 9.0, compared with the EC_{50} at pH 8.0, was observed.

The accumulated analysis of variance from the regression analysis indicated that there was not a significant difference in bacterial response between experiments (P=0.436).

3.5 Summary of results from Cr(VI) toxicity curves

Figures 3.5 to 3.9 presented the experimental and modelled concentration-response curves for the effect of Cr(VI) on the luminescence of *E. coli* pUCD607 from pH 4.5 to pH 9.0. All fitted models are plotted in Figure 3.10.

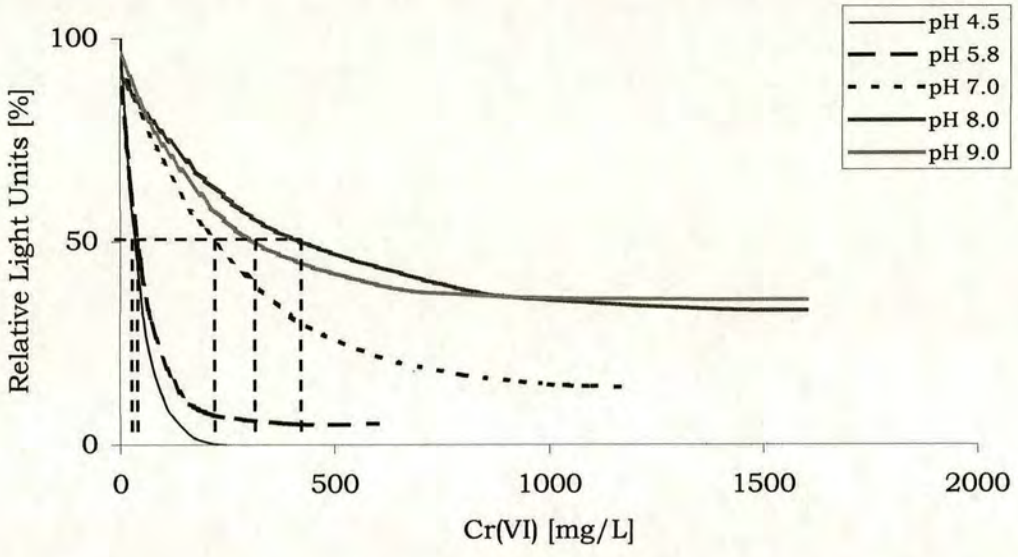


Fig. 3.10 Effect of pH on Cr(VI) toxicity to *E.coli* pUCD607. Exponential models representing Cr(VI) toxicity to *E. coli* pUCD067 at different pHs. EC_{50} values are indicated by interpolation.

The fitted models in Figure 3.10 showed that EC values and the shape of the curves were different at different pH. It was evident that:

- the toxicity of Cr(VI) to bacteria increased with decreasing pH, *e.g.* the concentrations of Cr(VI) necessary to reduce the light production by 50% tended to be higher at higher pH. Calculated EC_{50} values for pH 4.5, 5.8, 7.0, 8.0 and 9.0 were 34, 41, 218, 417 and 311 mg/L Cr(VI), respectively;

- the asymptotic behaviour of the concentration-response curves increased with increasing pH, *i.e.* at lower pH (4.5 and 5.8) the curve was more pronounced than at higher pH (8.0 and 9.0). Curves at pH 4.5 and 5.8 had a relatively similar shape. The curves at pH 8.0 and 9.0 were also similar. With increasing pH, the inflection of the curve was observed at higher %RLU values. From pH 7.0 upwards, the curves did not seem to tend to 0 %RLU and, therefore, it was not possible to calculate EC_{100} values from all concentration-response curves. From pH 7.0 upwards, even high concentrations of Cr(VI), which should be toxic to bacteria did not seem to completely inhibit the production of light.

The EC values were plotted against pH (Figure 3.11). The highest toxicity was observed below pH 6. The analysis of variance of the obtained EC values suggested a highly significant effect of pH on Cr(VI) toxicity ($P<0.001$).

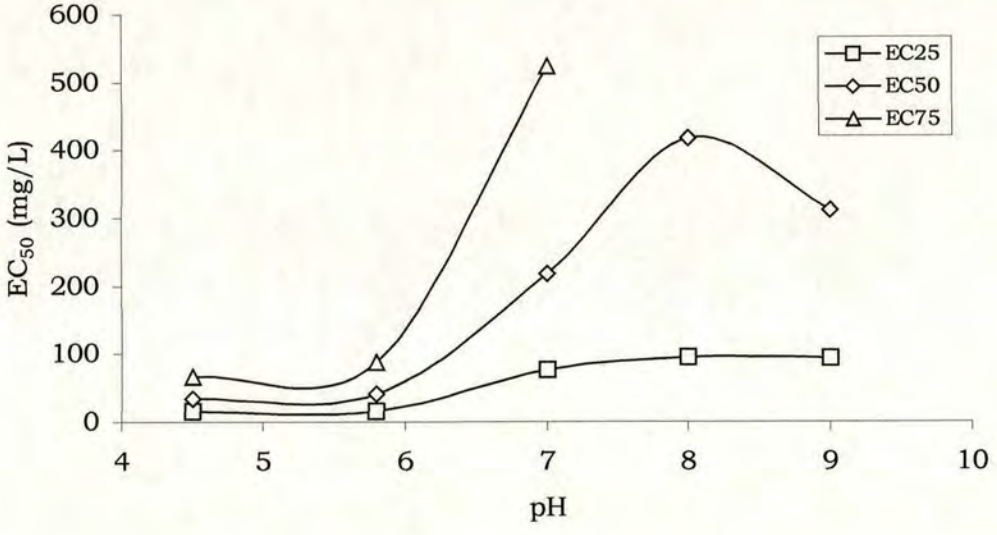


Fig. 3.11 Effect of pH on Cr(VI) toxicity to *E.coli* pUCD607. EC values at different pH. EC values were calculated using the exponential models obtained from the regression analysis.

3.6 Discussion

The EC values obtained from experimental and modelled concentration-response curves showed that pH had an effect on Cr(VI) toxicity to bacteria. Experiments in Section 3.3.4 showed that *E.coli* pUCD607 could perform vital functions in a pH range 6.5 to 8.5, without being subjected to excessive stress. Figure 3.11 showed that the toxicity of Cr(VI) decreased with increasing pH, but that the EC_{50} at pH 9.0 was lower than at pH 8.0, *i.e.* toxicity was higher at pH 9.0 than at pH 8.0.

The results from the experiments described in section 3.3.4, where the optimum pH range for *E. coli* pUCD607 (in 0.02M PO_4^{3-}) was investigated, are plotted in Figure 3.12, together with the EC_{50} values at different pHs.

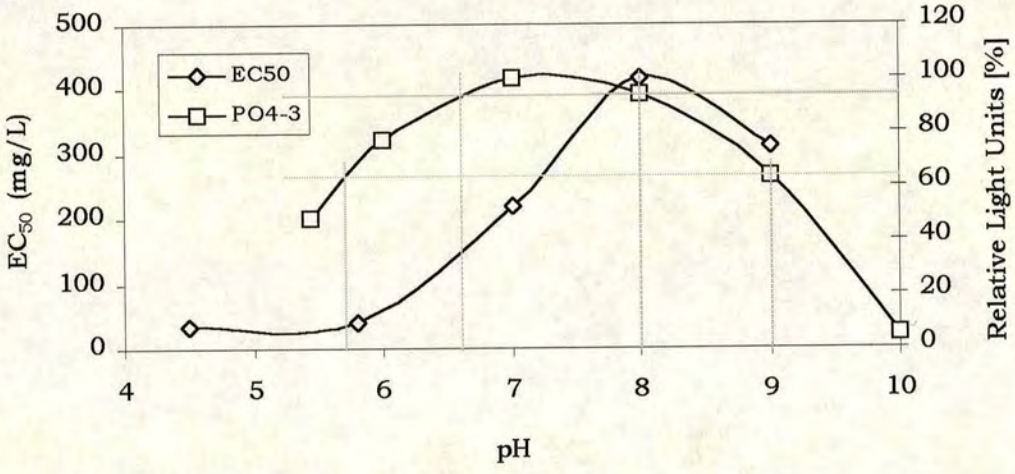


Fig. 3.12. Toxicity of Cr(VI) according to the EC_{50} values obtained from dose-response curves at different pHs compared with the toxicity of 0.02M phosphate solutions at different pH.

In Figure 3.12 the curve with squares represents the response of bacteria to 0.02M phosphate solutions at different pH. This curve shows that bacteria in 0.02M phosphate solutions at pH 8.0 would have a similar production of light as those in chromate-free solutions around pH 6.6, and that bacteria in 0.02M phosphate solutions at pH 9.0 would have a similar production of light as those in chromate-free solutions around pH 5.7. If the toxicity of the solutions containing Cr(VI) was mainly controlled by solution pH, similar EC_{50} values should be expected in solutions at pH 9.0 and pH 5.8, but according to the calculations they are different by an order of magnitude. Following the same reasoning, the toxicity of solutions at pH 4.5 and 5.8 should be very different. At pH 4.5 the production of light should be null or close to 0%, while at pH 5.8 it should be around 70%. Calculated EC_{50} values showed that the toxicity of Cr(VI) solutions at pH 4.5 and 5.8 was, however, very similar.

The effect of pH on the speciation of Cr(VI) was discussed in section 1.2.1., and Figure 1.1 showed the speciation diagram of Cr(VI) in 0.02M PO_4^{2-} (total Cr(VI)=4mM) with the distribution of the three major Cr(VI) species in solution: dichromate ($Cr_2O_7^{-2}$), hydrogen-chromate ($HCrO_4^-$) and chromate (CrO_4^{2-}), at a range of pH. Below pH 6.0, the predominant species was $HCrO_4^-$, followed by $Cr_2O_7^{2-}$, and at the lowest pH almost no CrO_4^{2-} was found. Over pH 6.5, the predominant species was CrO_4^{2-}, while the concentrations of $HCrO_4^-$ and $Cr_2O_7^{2-}$ decreased abruptly. At pH 8 and 9, at least 90% of Cr(VI) was found as CrO_4^{2-}.

Figure 3.13 and 3.14 compare the speciation diagram of Cr(VI) with the calculated EC_{50} values and the reciprocal of calculated EC_{50} values. Both curves suggest that the toxicity of Cr(VI) *to E. coli*

pUCD607 is related to the distribution of $HCrO_4^-$. The shape of the curve $1/EC_{50}$ is very similar to the $HCrO_4^{2-}$ curve and the difference between pH 8.0 and pH 9.0 solutions is not very obvious. The fractions of $HCrO_4$-, $Cr_2O_7^{2-}$ and CrO_4^{2-} at the studied pH were plotted against EC_{50} values in Figure 3.15. The fitted curves showed the highest correlation with $HCrO_4$-, followed by CrO_4^{2-}. The R values for the three curves are over 0.85, indicating a high correlation between speciation of Cr(VI) and toxicity, *i.e.* an increase in toxicity is observed with the increase of $HCrO_4^-$ and $Cr_2O_7^{2-}$ and a decrease in toxicity is observed with the increase of CrO_4^{2-} in solution.

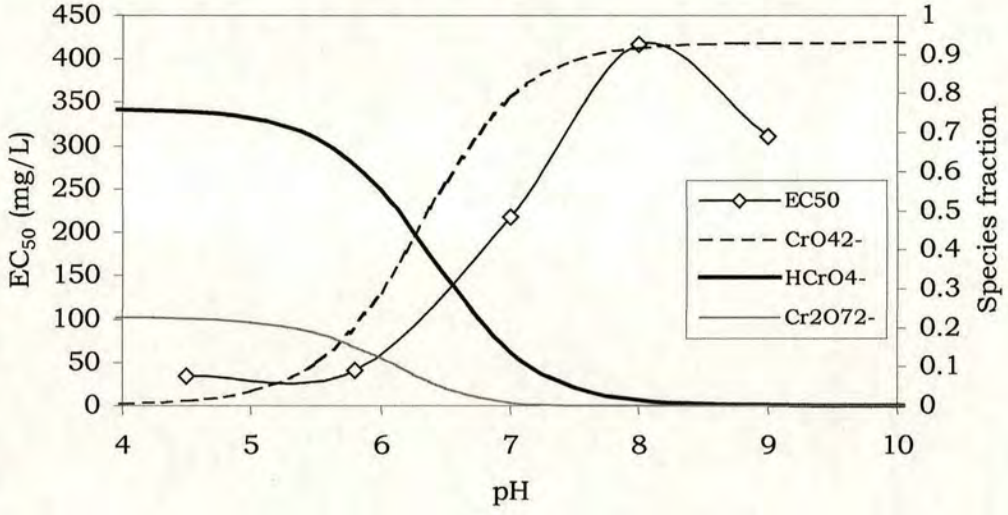


Fig. 3.13. Toxicity of Cr(VI) according to the EC_{50} values obtained from concentration-response curves at different pH and the three major species of Cr(VI) from the speciation diagram in Fig. 1.1.

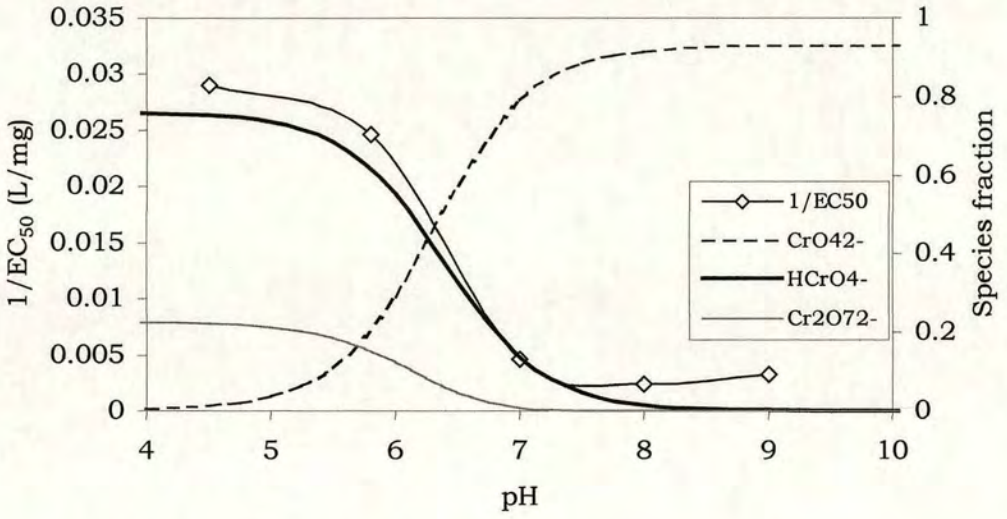


Fig. 3.14. Comparison of $1/EC_{50}$ and the three major species of Cr(VI) from the speciation diagram in Fig.1.1.

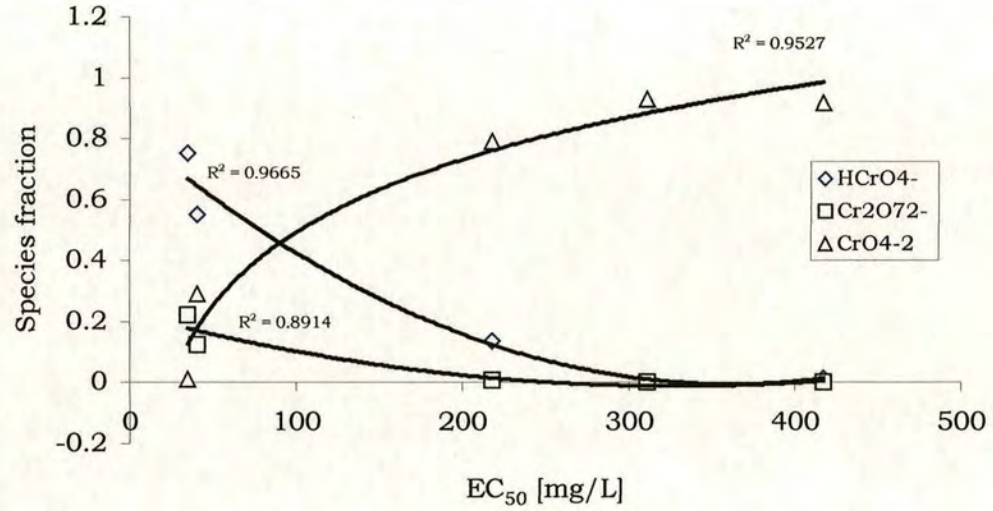


Fig. 3.15 Chromium(VI) species fractions at studied pH versus EC_{50} values.

Villaescusa *et al.* (1997) reported that hydrogen chromate was more toxic to *P. phosphoreum* than chromate. They used K_2CrO_4 in 2% NaCl solutions in the pH range 4.6 to 9.3 and an exposure time of 15

minutes. They also observed the highest toxicity at lower pH and a decrease in toxicity with the increase of pH until pH 7.0. EC_{50} values reported at pH 4.6, 5.9, 7.0 and 9.3 were 23, 112, 584 and 480 mg/L Cr(VI), respectively.

All EC_{50} values reported by Villaescusa *et al.* (1997), for *P.* phosphoreum, are higher than those obtained in these experiments with *E. coli* pUCD607, probably due to the difference in microbial species. Different sensitivity to heavy metals has been reported previously in luminescent biosensors with different species. Paton *et al.* (1997) found that *Rh. leguminosarum* bv *trifolii* was more sensitive to Cd but less sensitive to Zn, Cu and Ni compared with *Ps. fluorescens.*

García *et al.* (1994) investigated the toxicity of $K_2Cr_2O_7$ solutions to *P. phosphoreum* after a 15 minute exposure time. They reported an EC_{50}=375 μM $K_2Cr_2O_7$ (39 mg/L Cr(VI)). The pH was not specified, but if the pH of the solutions was not adjusted, it should have been around 4.5 to 5.0. The EC_{50} value reported by García *et al.* (1994) is closer to the EC_{50} values obtained in this work, at low pH, for *E. coli* pUCD607.

That hydrogen chromate is the most toxic Cr(VI) species in solution could be the result of this species being a stronger oxidising agent than chromate (Kimbrough *et al.*, 1999). Dichromate salts have been long used as oxidants in organic chemistry.

Chromium (VI) (as hydrogen chromate) appears to be less toxic to bacteria than other heavy metals, *e.g.* Campbell *et al.* (2000) reported on the toxicity of Cd, Cu and Zn to *E. coli* pUCD607, in solutions at pH 5.5 with 20 minutes exposure. They obtained EC_{50}

values of 1.85, 2.24 and 5.92 μM for Cd, Cu and Zn, respectively. Using *Rh. Leguminosarium* bv. *Trifolii* F6 pUCD607, Paton *et al.* (1997) reported EC_{50} values of 0.06, 0.42, 0.48 and 0.94 mg/L for Cd, Cu, Ni and Zn, respectively.

Chromium (VI) in the environment normally comes from anthropogenic inputs (Kimbrough, 1995); therefore concentrations in environmental samples will depend on the extent of those inputs. For example, Thomas *et al.* (2001) have reported high concentrations of Cr(VI) in soil pore waters (80 mg/L) and groundwaters (30 mg/L) in sites contaminated with chromite ore processing residue. They also reported that the pH of these solutions were as high as 12.5. The toxicity of samples with such high pH will most likely be controlled by pH rather than Cr(VI) contents or speciation.

Experiments in this chapter have demonstrated that Cr(VI) is toxic to different degrees to *E. coli* pUCD607, depending on the pH of the matrix. In principle this biosensor could be used to investigate and compare the toxicity of Cr-contaminated environmental samples.

3.7 Conclusions

From the experiments presented in this chapter, the following conclusions can be drawn:

- Cr(VI) toxicity to *E. coli* pUCD607 was mainly influenced by Cr(VI) speciation, with hydrogen chromate ($HCrO_4^-$) being the most toxic species.

- Within the optimal pH range for *E. coli* pUCD607, solution pH plays an important (but probably indirect) role in Cr(VI) toxicity, because it controls the speciation of Cr(VI).

- Important pH effects were observed which should be considered to be additive to Cr(VI) toxicity above and below optimum bacterial pH.

- Bioassays can determine the toxicity of solution systems taking into account all possible factors contributing to toxicity.

- The biosensor E. coli pUCD607 might be useful to investigate and compare the toxicity of Cr-contaminated environmental samples.

Chapter 4

Field Investigations

4.1 Introduction

A vast area of SE Glasgow and neighbouring South Lanarkshire, Scotland has been infilled with chromite ore processing residue (COPR) arising from a former chromium works (J.J. White's chemical works), in operation from *ca.* 1830 to 1968. Soils in the studied sites are a mixture of the highly alkaline COPR and organic soils. High pH of up to 12.3 has been reported (Thomas *et al.*, 2001) and the soils also contain high concentrations of Cr (3.6 % w/w) and Cr(VI) (0.85 % w/w), which have been leached to groundwaters resulting in Cr(VI) concentrations of up to 30 mg/L (Farmer *et al.*, 1999).

Chromium works traditionally produced chromates by using the air roasting (oxidation) of chromite ore (average composition: Cr_2O_3, 35%; Fe_2O_3, 20%; Al_2O_3, 15%; FeO, 12%; MgO, 12%) with alkali carbonate and calcium oxide (lime). This method, known as the high lime method, resulted in large quantities of waste containing alkaline residues such as $CaCrO_4$ (Kimbrough, 1999). It has been suggested that insoluble chromates such as $CaCrO_4$ are responsible for the carcinogenic effects of chromium-contaminated solids (Gochfeld, 1991).

The experiments in Chapter 3 showed that the biosensor *E. coli* pUCD607 was sensitive to Cr(VI) in solutions prepared in the laboratory and that this biosensor could potentially be used to determine the toxicity of Cr-contaminated environmental samples. Therefore, soil samples of one of the COPR-contaminated sites mentioned above were taken and their toxicity was studied using chemical and biological analyses.

4.2 Objectives

- To investigate the use of chemical analyses and the biosensor E. coli puCD607 in determining and explaining the toxicity and bioavailability of Cr in Cr-contaminated environmental samples.

- To investigate the use of luminescent *E. coli* pUCD607 as a rapid biosensor to measure the toxicity of Cr-contaminated environmental samples.

- To compare the toxicity to *E. coli* pUCD607 of synthetic solutions of Cr with the toxicity of Cr-contaminated environmental samples.

4.3 Hypotheses tested

- The use of *E. coli* pUCD607 together with chemical analyses will provide information on the bioavailability of Cr in environmental samples.

- Cr(VI) in environmental samples will be the major contributing factor on the toxicity of Cr-contaminated environmental samples.

- Matrix pH will be also a contributing factor on the toxicity of Cr-contaminated environmental samples.

- Synthetic solutions containing Cr will be more toxic to *E. coli* pUCD607 than Cr-contaminated environmental samples.

4.4 Site description and history

The study site selected was at Rutherglen Glencairn Football Club, South Lanarkshire. The site is located on the south-west side of Glasgow Road, west of the intersection with Quay Road, Rutherglen. It has an area of approximately 2.5 hectares, with made gound overlying sands, clays and silts, overlying carboniferous coal measures. The northeast half of the site comprises buildings used for the football and social club facilities, a turf football pitch, terracing and a stand. The south-west half of the site comprises open space and derelict land. The former chemical works was situated adjacent to the northern perimeter of the site (Dames and Moore, 1993).

The surface conditions vary across the site. Most of the site is flat, apart from the football terracing (Figure 4.1). The terracing was built from COPR and other residues from the chromium works. Apart from the grassed football pitch, other areas of the site are unsealed or covered by sparse vegetation, such as small trees and bushes (Figure 4.2). The remaining areas, such as the terracing have the COPR exposed. Even though the terracing is not officially in use any more, the football pitch is still used. Access to the site is easily gained from the main road and local children often use it as a playing ground. The site has also been used to deposit unwanted building materials.

Fig. 4.1 View of COPR terracing at Rutherglen Glencairn Football Club.

Fig. 4.2 View of derelict COPR-contaminated land adjacent to the football pitch (south-west side) at Rutherglen Glencairn Football Ground

4.5 Sampling strategy

4.5.1 Soil samples

Sixteen soil samples were collected from a 1.2 ha area, immediately adjacent to the football pitch. The sampling area included the football terracing, but excluded the football pitch.

A stratified random sampling design was used, taking samples at random positions from a grid of 15 (25 x 25 m) squares and recording their spatial coordinates relative to the established origin (eastern corner) (Figure 4.3). Samples were taken at different depths, depending on the existence of concrete-like layers, from 10 to 40 cm. In one grid, two samples were taken from the same point, one at 10 cm and the other at 30-40 cm.

The sampling was carried out on October 6th, 1998. Approximately 3 kg of sample was taken at each sampling point at variable depths from 80 x 80 cm square holes. Samples were placed in double polyethylene bags and kept in a cooled box for transportation to the laboratory, where they were stored at 4°C until analysed (Licona Manzur *et al.*, 2001).

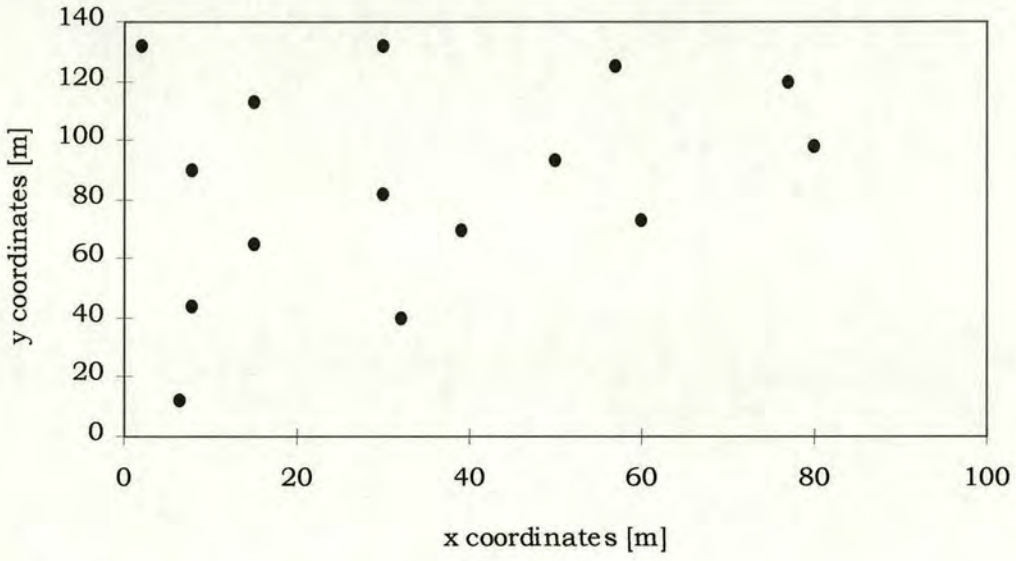


Fig. 4.3 Sampling design showing the spatial distribution of sampling points in COPR-contaminated site at Rutherglen Glencairn FC.

4.5.2 Groundwater samples

Groundwater samples were collected from three former COPR disposal sites, Duke's Road Playing Fields (Site 2), Rutherglen Glencairn Football Club (Site 4) and Rosebery Park (Site 7):

- Site 2 (6.5 ha) was formerly a quarry and now comprises former football pitches, a playground and open space. It consists of made ground overlying sandy clay overlaying carboniferous coal measures, with chemical waste infill material to depths of 2-10 m and groundwater 3-11 m below ground level.

- Site 4 (described in Section 4.3) has groundwater levels at 1.7-7.4 m from the surface, with borehole BH401 adjacent to the

terraces and BH402 a steep embankment leading down to a railway line.

- Site 7 (1.5 ha) is an infilled clay pit, now comprising a disused football ground, terraces and stands, with made ground overlying alluvial sands and clays above carboniferous coal measures. Groundwater lies at approximately 2 m below the ground level (Bewley *et al.*, 2000).

Groundwater samples from boreholes BH201, BH202, BH204 (Site 2), BH401, BH402 (Site 4) and BH702 (Site 7) were collected on September 10th, 1998. They were obtained using a 1-L polyethylene bailer (Geotechnical Instruments Ltd, UK). Each borehole was purged by removing three well volumes of water before collection of a representative sample. Samples were filtered on site through 0.45 μm membrane filters (PALL Gelman, UK) into acid-washed polyethylene bottles. A further 1 L of unfiltered sample was obtained for subsequent filtration in the laboratory. Samples were kept in cold boxes for transportation to the laboratory, where they were analysed for Cr(VI) immediately and stored at 4°C for further analyses.

4.6 Measurements and analytical procedures

The extent of vegetation at each sampling point was recorded using a scale of 0 to 3, where 0 represented no vegetation cover and 3 heavy vegetation cover.

In the laboratory, soil samples were sieved through a 4 mm mesh and soils <4 mm were stored in double polyethylene bags at 4°C until use.

Groundwater samples were filtered through 0.45 μm membrane filters immediately after collection and the sample was divided in two. One portion of the sample was acidified with 2M HNO_3 and stored air-tight polyethylene bottles at 4°C until analysed by ICP-OES. The other portion was stored in air-tight polyethylene bottles, but was not acidified, and was used to determine Cr(VI) and toxicity to biosensors. Chromium (VI) analysis by the colorimetric method described in Section 2.2.5 was carried out on the day of sample collection. The non-acidified groundwater was stored in air-tight polyethylene bottles at 4°C until its toxicity was tested.

Soil samples were:

1. analysed for soil moisture and pH (Section 2.1.1 and 2.1.3);

2. extracted with phosphate buffer in order to determine soluble and exchangeable Cr(VI) and other elements (Section 2.2.4);

3. ashed to calculate the amount of organic matter by LOI (Section 2.1.4);

4. digested with *aqua regia* to determine pseudo-total element concentrations (Section 2.2.2);

5. digested under alkaline conditions to determine total Cr(VI) (Section 2.2.3);

6. centrifuged to extract the soil pore waters (Section 2.2.1);

7. analysed for dissolved total elemental and Cr(VI) concentrations (Sections 2.2.6 and 2.2.5).

Soil samples were used as found in the field, *i.e.* not dried, in order to account for conditions as close as possible to reality. Unless there were obvious signs of organic matter, the solutions were acidified with 2% HNO_3 prior to ICP-OES analysis.

The toxicity of those solutions below pH 9.0, *i.e.* phosphate extracts and groundwater samples, was tested using *E. coli* pUCD607, as described in Section 2.3.1. A 0.02M phosphate solution at pH 7.0 was used as a control against which all samples could be compared. Also, 0.02M phosphate solution controls at the same pH as each sample tested were used. Light output was measured for phosphate extracts and controls, and compared.

4.7 Results from the analysis of soil samples

In general, a high variation in the characteristics and composition of soil samples was found. The description and physicochemical properties of the collected samples are included below.

4.7.1 Sample description and vegetation cover

The material sampled was highly heterogeneous, containing COPR, soils and building materials in different measure. This material could not be defined as soil, but for ease, the notation "soil sample" will be used when referring to the solid material collected.

In general it was not possible to identify soil profiles as such, but in some cases three irregular layers at varying depths and widths could be discerned: a thin layer of organic soil, a hardened cement-like crust and a zone where loose COPR was predominant (Figure 4.4). As soil samples were taken at different depths and depending on the ease of sampling, COPR might have been present to different degrees in the sample.

Fig. 4.4 COPR-contaminated land adjacent to the football pitch (south-west side) at Rutherglen Glencairn Football Ground. Photograph of 60 x 60 cm square, 5 cm deep excavation, showing thin layer of organic soil on the surface, over a hard, cement-like layer, over loose COPR.

Soil samples could be divided visually according to their ratio of "organic soil" to COPR. Soil samples containing high amounts of COPR had characteristic yellow-green *cumuli*, sometimes accompanied by red *cumuli*. When greater amounts of COPR were present, the soil samples were in general coarser in texture and had higher moisture contents than soil samples of lesser COPR content. A physical particle analysis was not possible, due to the

heterogeneity of the samples. In this case a particle classification would have been erroneous.

Vegetation (primarily grasses and moss) was observed mainly in association with deeper organic topsoil, while in areas where the COPR was exposed (either as a hardened layer or as loose material), vegetation was absent for several meters around.

A more detailed description of the soil samples, their spatial coordinates and vegetation cover are included in Table 4.1.

4.7.2 Physicochemical properties of soil material

All soil analyses were carried out using the < 4mm fraction. Unless indicated otherwise, fresh (not dried) sample was used.

4.7.2.1 Moisture content, organic matter and pH

The variation in the soil samples was high. A wide range of pH, moisture and organic content (Figure 4.5) was found:

- The soil samples were, in general, alkaline with pH values varying between 7.7 and 11.6.

- The moisture content of soil samples varied between 7.9 and 50%.

- The organic matter content of the soil samples varied between 4 and 25%.

Table 4.1 Description of soil samples collected from Rutherglen Glencairn FC.

Soil	Sample depth [cm]	x coordinates[+] [m]	Y coordinates [m]	Description	Vegetation*
1	10-20	6.5	12.5	Soil mixed with grey material containing yellow cumuli	0
2	10	32	40	More soil-like appearance mixed with building materials (brick, etc.)	1
3	10-20	8	44	Sample area surrounded by hard material, top layer light brown, middle layer light pink, lower layer dark brown	1
4	10-15	39	70	Top blue-violet layer, followed by a grey layer with yellow, blue-green and red cumuli	0
5	10-30	60	73	Material with clay texture, including a hard violet layer	0
6•	30-40	60	73	Top soil covering sample 5. Light brown material	2
7	5	15	65	Dark soil over a brick layer	2
8	10-15	50	93	Dark brown soil, no profile distinguishable	3
8	10-20	80	98	Brown-grey material with building materials and some soil	0
10	15-20	30	82	Loose material at the surface similar to soil with a hard layer with yellow-green cumuli	1
11	15-20	8	90	Brown soil-like material with red brick particles and yellow cumuli, very moist and with carbonaceous residues	2
12	15-20	15	113	Brown soil with an orange layer	2
13	15-20	30	132	COPR grey-brown with yellow cumuli	0
14	15-20	77	120	More similar to building waste material with little organic soil	1
15	15-20	57	125	Hard grey material with yellow cumuli	3
16	10-20	2	132	Brown, fine particulate material, dry	2

[+] Coordinates were measured from an established zero, indicated in Figure 4.3

* Refers to degree of vegetation on the surface cover from where the sample was taken, using a subjective scale where 0=no vegetation, 1=slight vegetation, 2=medium vegetation and 3=dense vegetation.

• Sample 6 was taken from the same spot as sample 5 but at a different depth. A hardened cement-like layer separated the samples

In general, it was observed that samples that contained higher amounts of COPR had higher pH and moisture and lower organic matter content. However, the values for organic matter content by LOI may have been influenced by loss of CO_2 from calcium carbonate present in COPR, as a result of the high lime process.

4.7.2.2 Pseudo-total metal content

The variation in the chemical composition of soil samples was high. Samples were analysed for Al, Ba, Ca, Cr, Cu, Fe, Mg, Mn, Si and Zn. According to the concentrations found, elements could be divided into three groups:

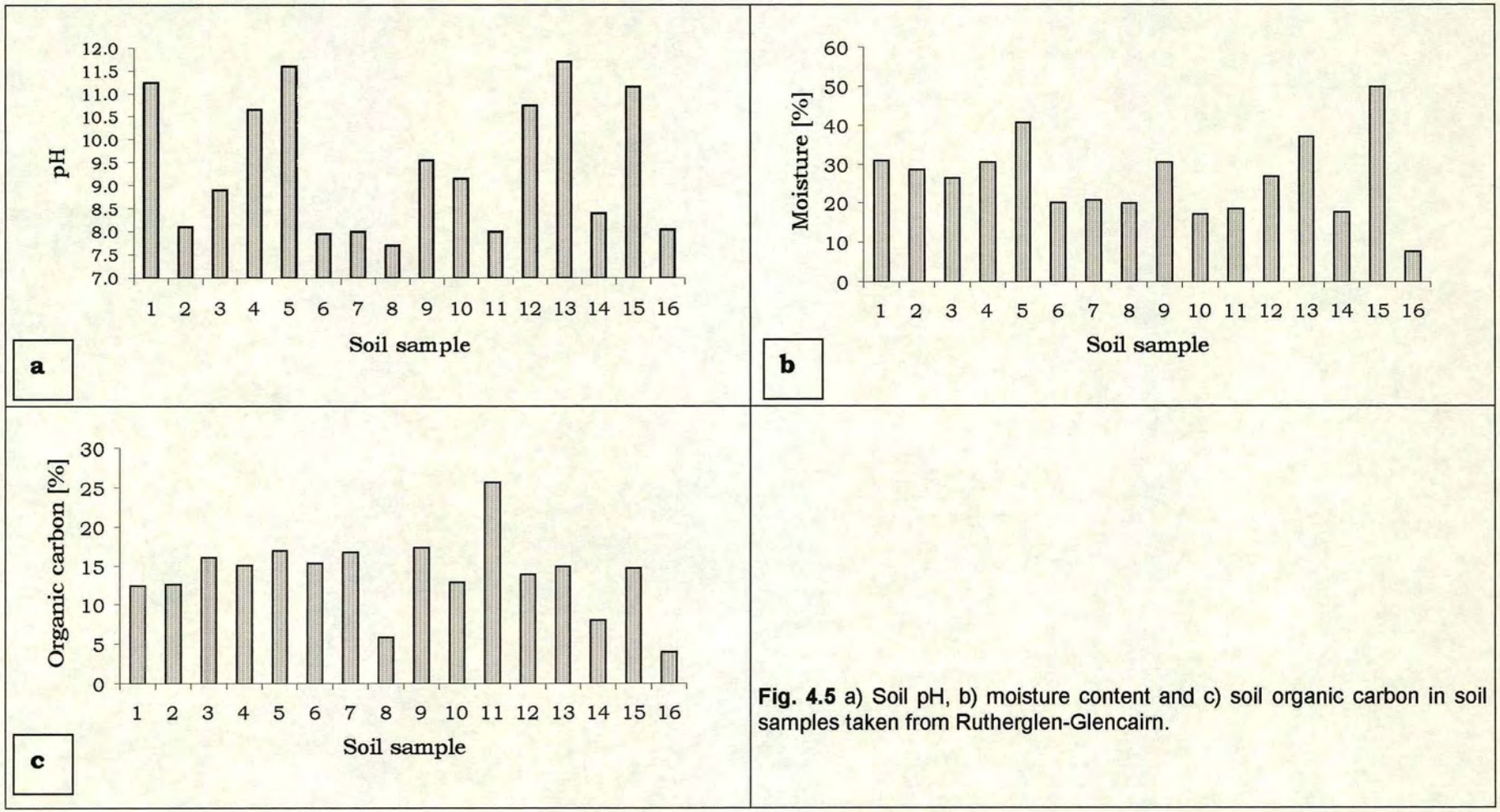


Fig. 4.5 a) Soil pH, b) moisture content and c) soil organic carbon in soil samples taken from Rutherglen-Glencairn.

- Elements present in higher concentrations in soils (Figure 4.6), including Ca (1.1-20.5% (w/w)), Mg (0.5-19.9% (w/w)) and Fe (0.5–12.0% (w/w)). Samples with the highest concentrations of these elements were 1, 4, 5, 12, 13 and 15.

- Total Cr, which was also found in relatively high concentrations in some samples. The range of Cr concentration varied between 0 and 3.1% (w/w) (Figure 4.7). The highest concentrations of Cr were found in samples 1, 4, 5, 12, 13, and 15 (the same as those for the highest concentrations of Ca, Mg and Fe).

- Elements present in low concentrations in soils (Figure 4.8), including Al (0.05–45.8 g/kg), Ba (10–500 mg/kg), Mn (80-2800 mg/kg), Si (0–3000 mg/kg) and Zn (40–1800 mg/kg). Three samples were found to contain high amounts of Al, but for no apparent reason.

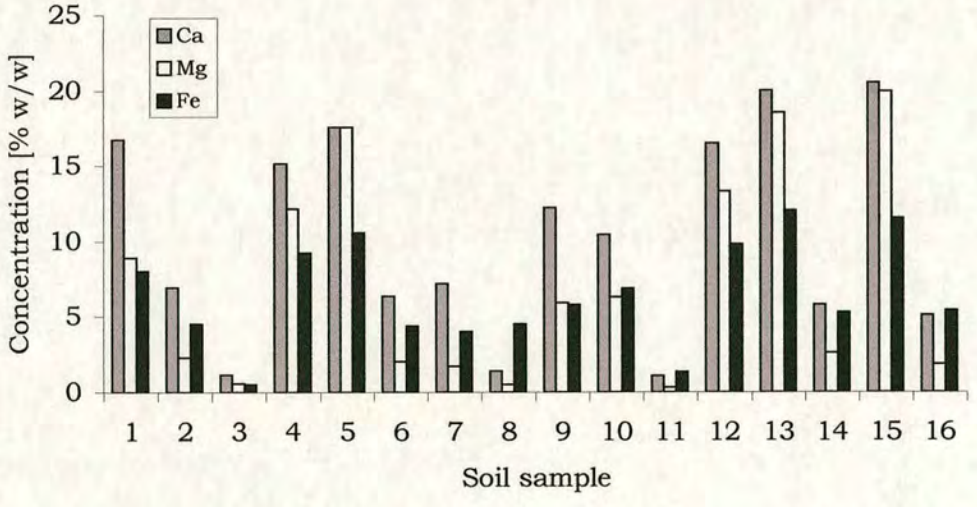


Fig. 4.6 Elements in higher concentrations in Rutherglen Glencairn soil samples, as determined in *aqua regia* digestions.

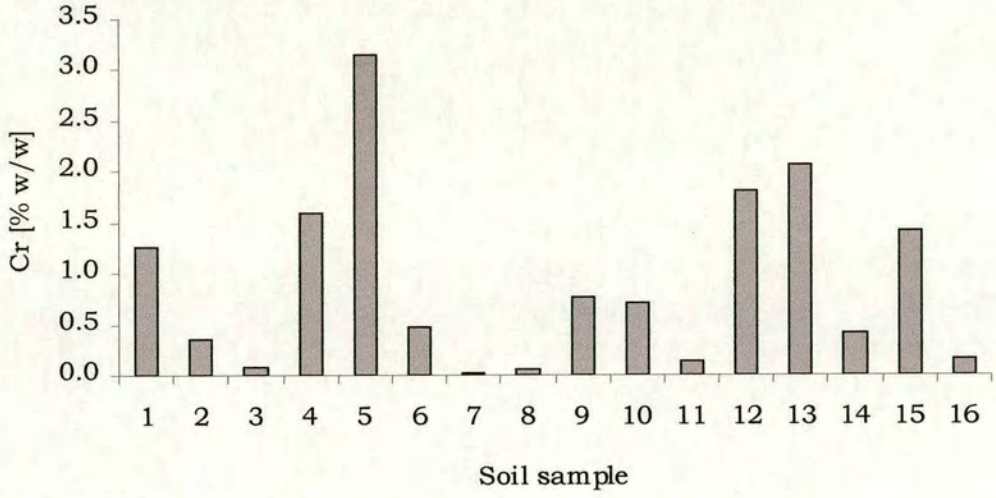


Fig. 4.7 Total Cr in Rutherglen Glencairn soil samples, as determined in *aqua regia* digestions.

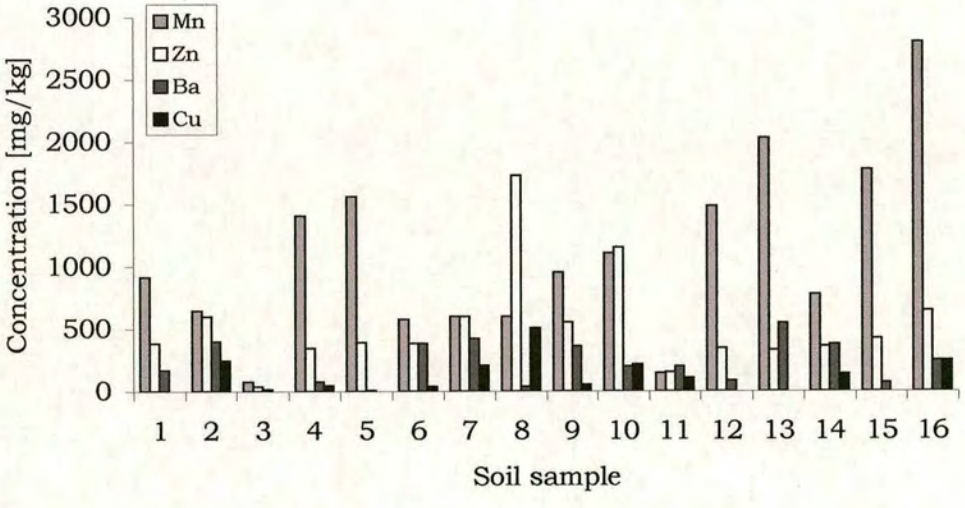


Fig. 4.8 Elements present in lower concentrations in Rutherglen Glencairn soil samples, as determined in *aqua regia* digestions

4.7.2.3 Total Cr(VI) in alkaline digestions

Total Cr(VI) was found in alkaline digestions at levels between 0 and 0.4% (w/w) (Figure 4.9). Figure 4.10 shows that only a small percentage of Cr in the samples was in the hexavalent form, compared with total Cr (0-3.1% (w/w)) and Cr(III) (0-2.7% (w/w))[1]. Samples showing the highest concentrations of total Cr(VI) corresponded with the samples showing the highest concentrations of total Cr (samples 1, 4, 5, 12, 13 and 15).

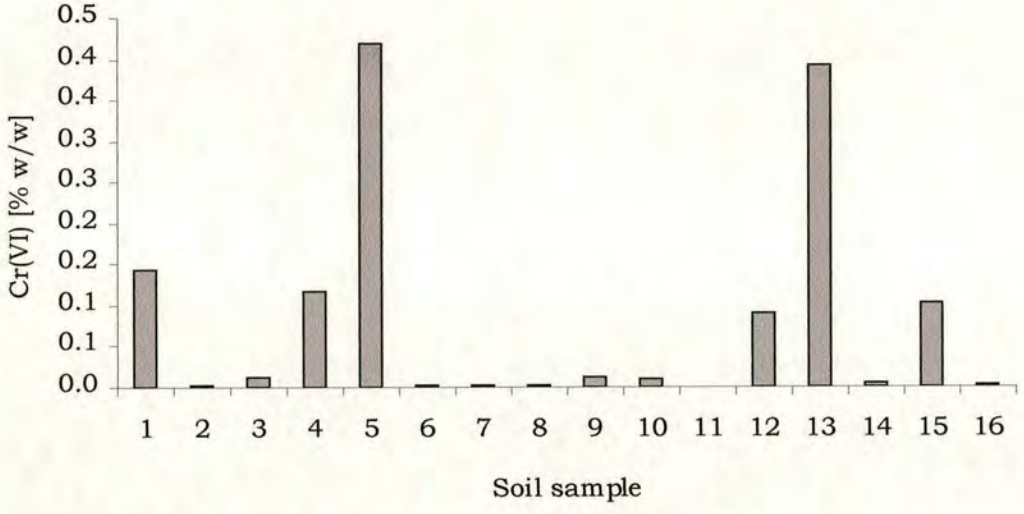


Fig. 4.9 Chromium (VI) in Rutherglen Glencairn soil samples, as determined in alkaline digestions.

At least six soil samples were relatively high in Cr(VI). Samples 5 and 6, which were taken from the same place but at different depths, had very different Cr(VI) concentrations. Sample 6, which was overlying the hardened layer (Table 4.1), had very low concentrations

[1] The amount of Cr(III) was obtained from the mathematical difference between total Cr from *aqua regia* digestions and Cr(VI) from the alkaline digestions.

of Cr(VI) compared with sample 5, which was below the hardened layer and contained mostly COPR.

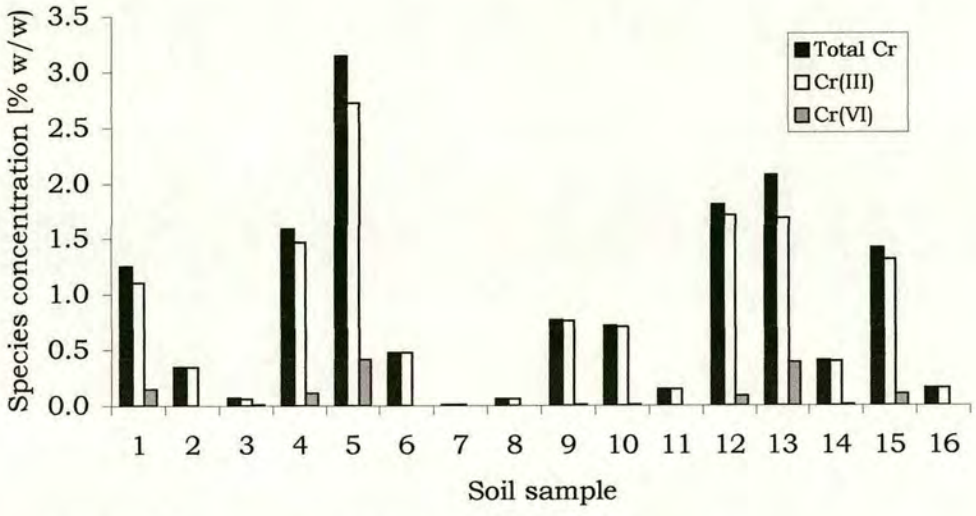


Fig. 4.10 Chromium species in Rutherglen Glencairn soil samples.

4.7.3 Phosphate extractions

Phosphate extracts were obtained from soil samples as described in Section 2.2.4 and were analysed both by ICP-OES (Section 2.2.6) and the diphenylcarbazide method (Section 2.2.5).

4.7.3.1 Chemical composition and pH of phosphate extracts

As phosphate extracts were going to be assayed with *E. coli* pUCD607, it was important to determine their pH and elemental composition. Figure 4.11 shows the pH of soil phosphate extracts which, in general, was much lower than that of water extractions (data not shown).

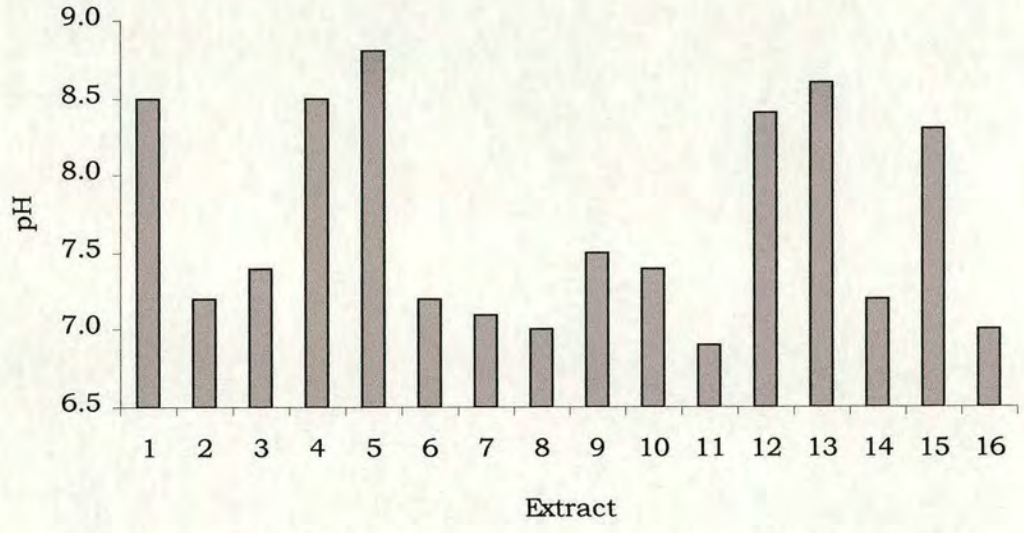


Figure 4.11 pH of phosphate extracts from sixteen soil samples from Rutherglen Glencairn.

The extracts were analysed by ICP-OES for Al, Ca, Cr, Cu, Fe, Mg, Mn, Si and Zn. A high variation in the chemical composition of phosphate extracts was found. Based on concentration, it was possible to distinguish two groups of elements:

- Major elements (Figure 4.12a and 4.12b), including Ca (2-20 mg/L), Cr (0-11 mg/L), Mg (1-19 mg/L), and Si (1-23 mg/L); or calculated in a solid basis Ca (134-946 mg/kg), Cr (2-702 mg/kg), Mg (75-1194 mg/kg) and Si (49-1802 mg/kg). Sample 15 showed a high amount of Si compared with other samples, maybe due to analytical error.

- Minor elements (Figure 4.12c), including Al (0-37 mg/kg), Fe (0-38mg/kg) and Zn (0-3 mg/kg). Copper and Mn concentrations were very low and in most samples below detection limits (Section 2.2.6).

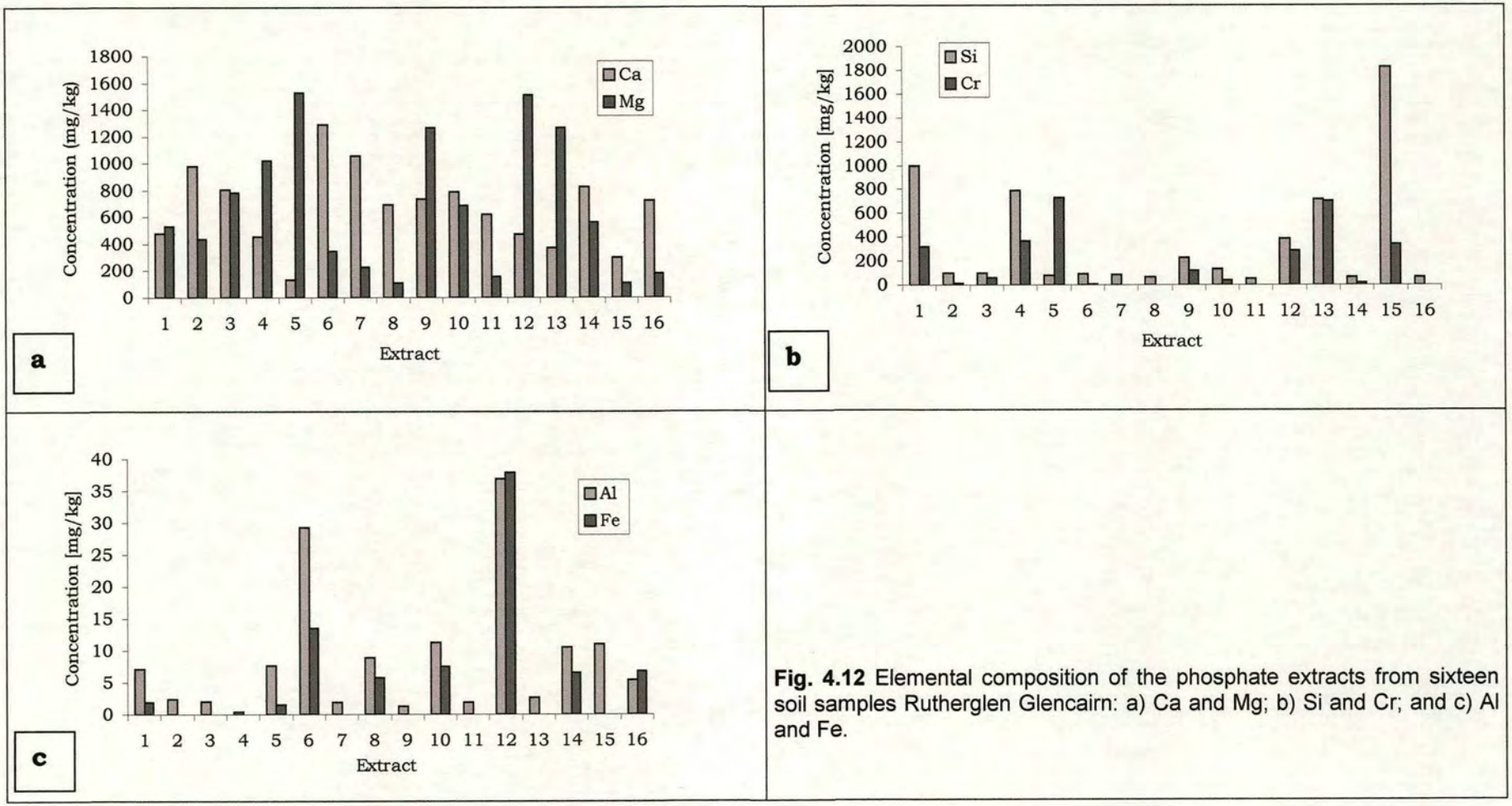


Fig. 4.12 Elemental composition of the phosphate extracts from sixteen soil samples Rutherglen Glencairn: a) Ca and Mg; b) Si and Cr; and c) Al and Fe.

4.7.3.2 Soluble and exchangeable Cr(VI) in phosphate extracts

Figure 4.13 shows that most chromium in the phosphate extracts was present as Cr(VI). The slight differences in some samples could be attributed to analytical error. Concentrations of Cr(VI) in the extracts ranged between 0.02 and 11 mg/L Cr(VI) (3-700 mg/kg soil).

Different percentages of Cr(VI) were recovered by the 0.02M phosphate extraction from different samples, probably due to the form in which Cr was bound/sorbed to other soil components. Organic matter, Al, Ca and Fe concentrations in the soils could be considered important factors contributing to the sorption/retention of Cr(VI) in soil matrices (Kimbrough *et al.*, 1999; Ainsworth *et al.*, 1989), therefore controlling Cr(VI) availability.

In order to determine the factors that influenced the difference in recovery of Cr(VI) among samples, a correlation matrix was calculated. Some correlations were found with Cr(VI) percentage recovery in phosphate extracts and soil components, although they were not highly significant:

- positive with Si in soils (r=0.604, P=0.01);
- negative with Fe in soils (r=-0.511, P=0.05);
- positive with organic matter (r=0.410, P=0.1);
- no correlations were found with Ca and Al concentrations in soils.

Highly significant correlations (P=0.001) were found between the concentration of Cr(VI) in phosphate extracts[2] and the concentrations of other elements in soils. Positive correlations were found with total Cr in soils (r=0.946), total Cr(VI) in soils (r=0.969), soil pH (r=0.912), moisture content (r=0.748), Ca in soils (r=0.832), Fe(r=0.827) and Mg (r=0.902).

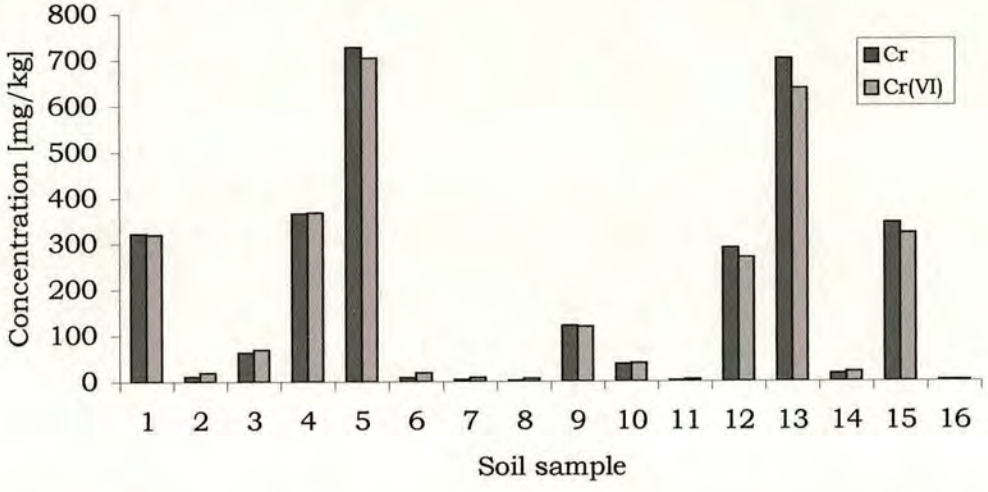


Fig. 4.13 Total Cr and exchangeable and soluble Cr(VI), as determined by ICP-OES (Cr) and the diphenylcarbazide method (Cr(VI)).

Besides the obvious correlation between Cr(VI) found in phosphate extracts with the amount of Cr and Cr(VI) in the soil samples, the soil pH might be the most important factor in controlling the presence of Cr(VI) in phosphate extracts, perhaps by allowing a higher dissolution of CrO_4^{2-} from mineral phases. Thomas *et al.* (2001) suggested that, in the site under study, CrO_4^{2-} was likely to be present as an exchangeable anion in the interlayer of the

[2] Referring to Cr(VI) in the extracts and not to the analytical percentage of recovery mentioned earlier on. These correlations could determine the factors that contribute to the concentrations of Cr(VI) in the extracts, but not the more complex interactions of phosphate buffer with Cr and other soil components that determine the recovery of Cr(VI).

hydrocalumite phase ($Ca_2(Al,Fe)(OH)_6(CrO_4)_{0.5}$). The positive correlations found between Cr(VI) in phosphate extracts and Ca, Fe and Mg could possibly indicate that the concentration of Cr(VI) in phosphate extracts depended on the amount of hydrocalumite in the soil sample.

Thomas *et al.* (2001) also suggested that it is possible that CrO_4^{2-} could substitute for silicate in the mineral structure of the hydrogarnet phase ($Ca_3(Al,Fe)_2(OH)_{12}$). Chromate in the hydrogarnet phase would probably be more tightly bound and therefore less available than CrO_4^{2-} in the hydrocalumite phase. It might be possible that the positive correlation found with Si and Cr(VI) recovery mentioned previously, could also indicate that most of the available CrO_4^{2-} comes from hydrocalumite.

4.7.4 Summary of main findings from the analysis of soil samples

- The variation in the physicochemical composition of soil samples was high.

- Samples contained different amounts of COPR, soil and building material.

- The pH of soil samples was alkaline, the highest pH found was 11.6.

- High concentrations of Ca, Mg and Fe were found in some samples (samples 1, 4, 5, 12, 13 and 15).

- High concentrations of Cr and Cr(VI) were found in some soil samples (samples 1, 4, 5, 12, 13 and 15).

- Only a small percentage of Cr was found as Cr(VI) in the soil samples.

- Phosphate buffer extracted different percentages of Cr(VI) in different samples and there was a significant correlation between Cr(VI) percent recovery and Si in soils.

- Concentrations of Cr(VI) in phosphate extracts seemed to be correlated to pH and concentrations of Ca, Mg and Fe, possibly related to the amounts of hydrocalumite (CrO_4^{2-} as exchangeable species) present in the soil samples.

4.8 Soil pore waters and groundwater samples

The chemical composition of extracted soil pore waters and collected groundwater samples was analysed by ICP-OES and the diphenylcarbazide method.

4.8.1 Extraction and analysis of soil pore waters

Soil pore waters were extracted from soil samples (Section 2.2.1) in their field moist state, but it was not possible to extract any solution from samples 14-16, which were too dry.

A wide range of pH was found (Figure 4.14). In general, the pH of the soil pore waters was higher than the pH of phosphate extracts (Figure 4.11). Soil pore waters 1, 4, 5, 12 and 13 were above pH 9.5.

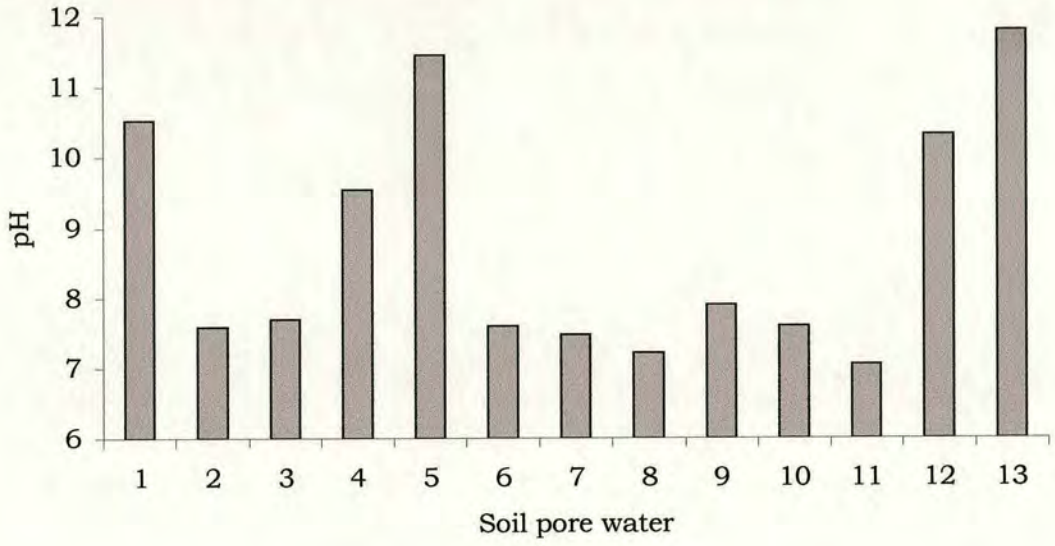


Fig. 4.14 The pH of soil pore waters extracted from Rutherglen Glencairn soil samples 1 to 13.

Major elements found in soil pore waters by ICP-OES were Ca (10-128 mg/L, Cr (0-43 mg/L), Mg (0-45 mg/L) and Si (0-62 mg/L). Aluminium, Cu, Fe, Mn and Zn concentrations were below detection limits (Section 2.2.6). The concentrations of Ca, Cr and Mg in solution were higher in soil pore waters than in phosphate extracts (Figure 4.15).

The highest concentrations of Cr in soil pore waters (Figure 4.15b) were found in the same samples as the highest concentrations of Cr in phosphate extracts, *i.e.* samples 1, 4, 5, 12 and 13. It is interesting to note that Mg concentrations in soil pore waters 1, 4, 5, 12 and 13 were lower than the concentrations in phosphate extracts (Fig. 4.15c), probably indicating that phosphate extracted Mg in other forms, apart from soluble ones.

Soil pore waters were also analysed for Cr(VI) using the diphenylcarbazide method (Section 2.2.5). Chromium in soil pore

waters was found entirely as Cr(VI) (Figure 4.16), as found in phosphate extracts. Concentrations of Cr(VI) ranged from 0 to 43 mg/L.

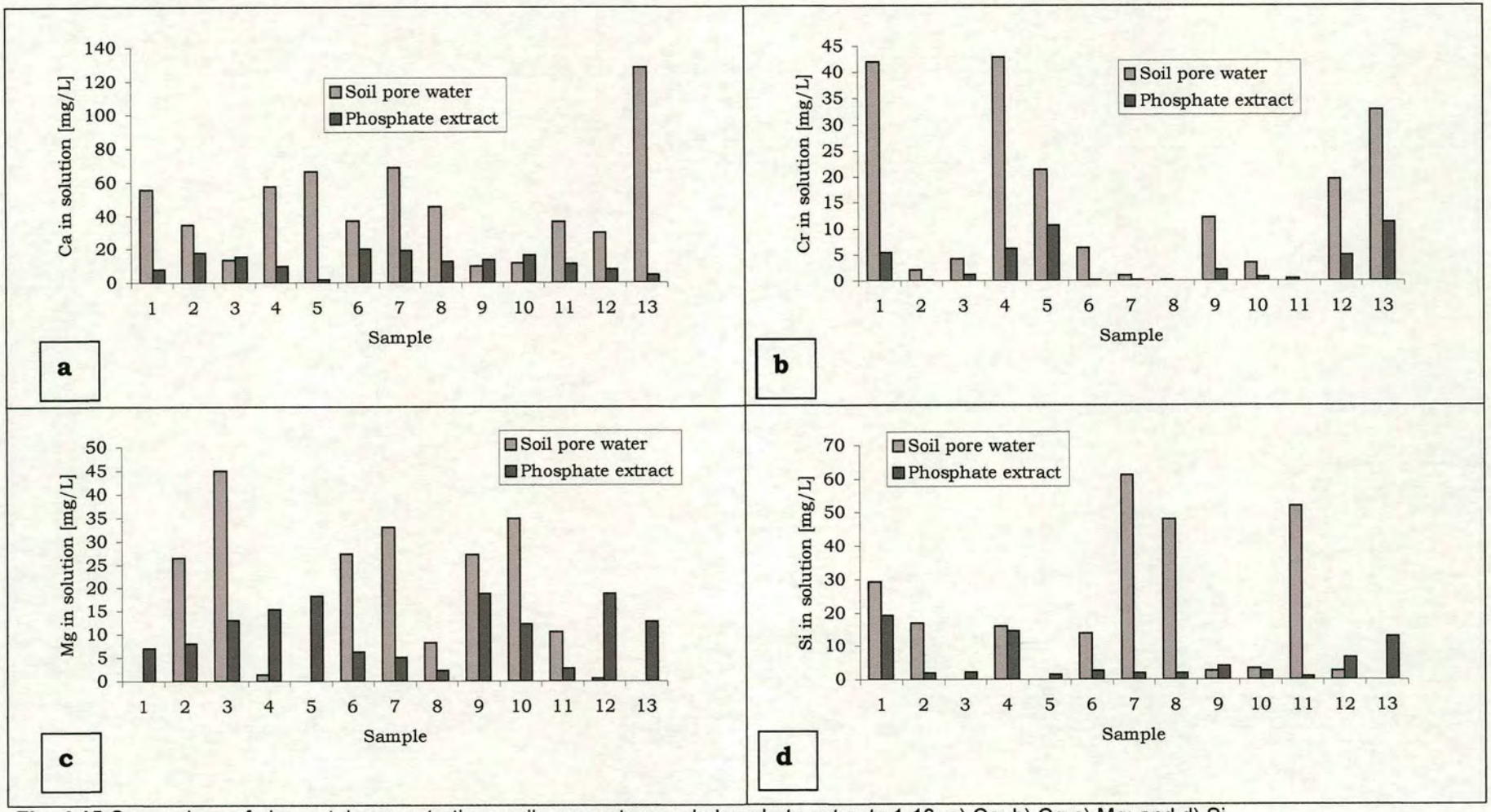


Fig. 4.15 Comparison of elemental concentrations soil pore waters and phosphate extracts 1-13: a) Ca; b) Cr; c) Mg; and d) Si.

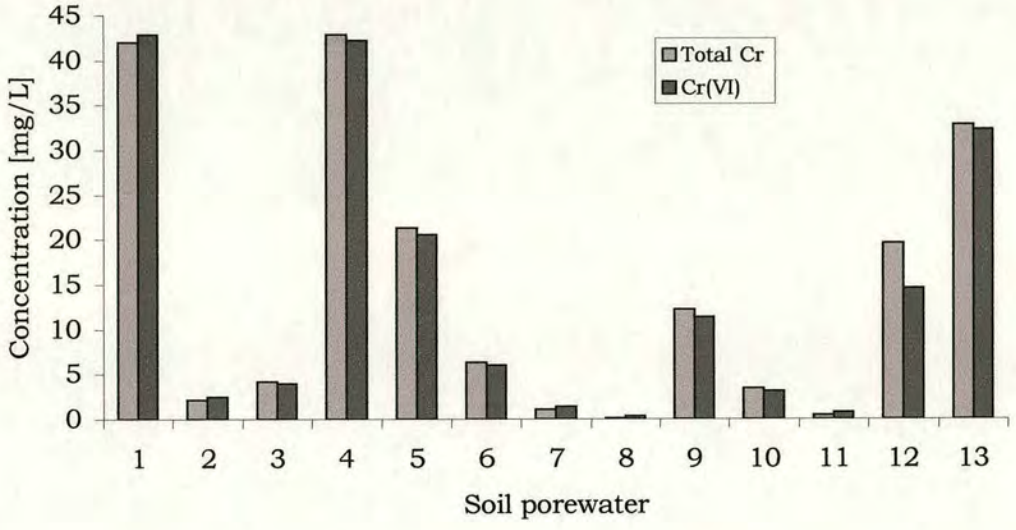


Fig. 4.16 Cr species in soil pore waters from Rutherglen Glencairn soil samples 1 to 13.

Soil pore waters that contained the highest concentrations of Cr(VI) were again 1, 4, 5, 12 and 13, but this time the highest concentrations of Cr(VI) were found in samples 1 and 4. The pore water and phosphate Cr(VI) values are compared in Figure 4.17.

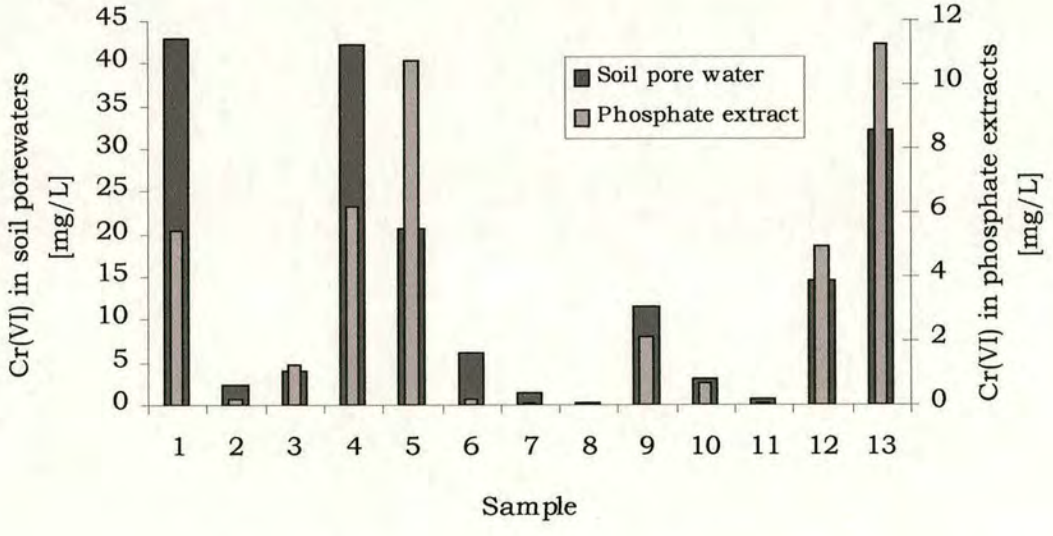


Fig. 4.17 Comparison of Cr(VI) concentrations in soil pore waters and phosphate extracts from Rutherglen Glencairn samples 1 to 13.

4.8.2 Analysis of Groundwater samples

The collected ground water samples were coloured. Samples from BH201, BH204 and BH702 were yellow, sample BH202 was orange and samples BH401 and BH402 were brown. They also presented different degrees of turbidity, therefore chemical analyses were performed on samples filtered through 0.45 μm membrane filters.

Once again, a high variation between samples was observed, with major elements being Ca (34-146 mg/L), Cr (0.01-30 mg/L) and Mg (0.2-66 mg/L) (Table 4.2). Both Dames and Moore (1993) and Farmer *et al.* (2002) found that the concentration of Cr(VI) in groundwater samples obtained from the same boreholes varied at different sampling times, with concentrations of Cr(VI) up to 90 mg/L.

Chromium was found as Cr(VI) in most of the groundwater samples, apart from, sample BH402, where there was a significant difference between total Cr and Cr(VI) (Figure 4.18).

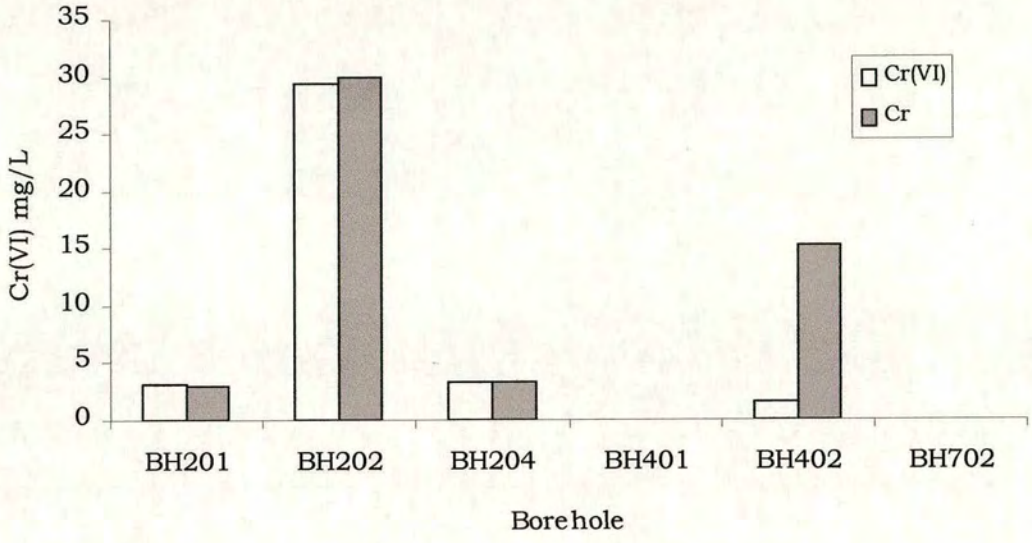


Fig. 4.18 Cr species found in six groundwater samples from three different COPR contaminated sites (Sampled 10/8/1999).

Table 4.2 Results for the analysis of groundwater samples from COPR contaminated sites (filtered through 0.45 μm membrane filters). Apart from pH and borehole and water depth, all units in mg/L.

Borehole	Borehole Depth (m)	Water depth (m)	pH on sampling date	Total Cr	Cr(VI)	Al	Ca	Fe	Mg	Mn	Cl^-	F^-	NO_3^-	SO_4^{2-}
BH201	13	3.2	7.2	3.04	3.23	0.15	122	<0.003	47	<0.001	39	3.6	9.5	90
BH202	17.7	9.1	7.7	30.0	29.4	0.14	82	<0.003	61	0.016	58	3.2	7.7	208
BH204	16.8	10.1	7.1	3.35	3.35	0.15	128	<0.003	61	<0.001	76	3.1	8.1	133
BH401	4.9	1.3	7.7	<0.01	<0.01	0.07	58	<0.003	66	0.43	44	3.1	7.3	52
BH402	6.2	2.5	12.5	15.2	1.63	9.6	34	3.5	0.2	0.12	32	5.6	7.5	35
BH702	14.5	3	7.5	<0.01	<0.01	0.2	146	<0.003	30	0.67	58	3.4	6.9	38

4.8.3 Summary of main findings in soil pore waters and groundwater samples

- It was only possible to extract soil pore waters from thirteen of the sixteen soil samples, because the samples were extracted at field moisture and some were too dry.

- There was a high variation in soil pore water pH. Samples with the highest content of Cr had the highest pH.

- In soil pore waters, all Cr was found as Cr(VI).

- Ground water samples had a high variation in chemical composition. The major elements were Ca, Mg and Cr, mainly as Cr(VI).

4.9 Biological analysis

4.9.1 Bioassays on phosphate extracts

The toxicity of soil phosphate extracts was tested using *E. coli* pUCD607. The bioassays were performed as described in Section 2.3.1. Experiments were divided into two sets, according to the type of control (100% RLU) used:

- Set 1 - 0.02 M phosphate solution at pH 7.0 was used as control. This set of experiments had the purpose of recording the toxicity of phosphate extracts arising from all possible contributing factors;

- Set 2 - 0.02 M phosphate solutions at the same pH as the individual phosphate extracts were used as controls. This set of experiments had the purpose of separating possible effects of pH from possible chemical composition effects on the toxicity of the phosphate extracts.

Each phosphate extract was tested with at least two different bioassays (using two different vials). In each experiment, phosphate extracts were tested in triplicate. The mean %RLU values from the experiments were used.

Results from set 1 experiments (Figure 4.19) showed that the most toxic phosphate extracts were 1, 4, 5, 9 and 15 with %RLU below 75. Extracts 1, 4, 5, 9 and 15 contained 5, 6, 10, 2 and 4 mg/L of Cr(VI), respectively. Sample 5 had the highest concentration of Cr and also presented the highest toxicity to bacteria.

Results from set 2 experiments (Figure 4.20) showed different relative toxicities. Phosphate extracts 7 and 8 were the most toxic, with <65% RLU, followed by phosphate extracts 9 and 11, with <75% RLU. Phosphate extracts 7, 8, 9 and 11 contained 0.1, 0.1, 2.1 and 0.1 mg/L Cr(VI), respectively.

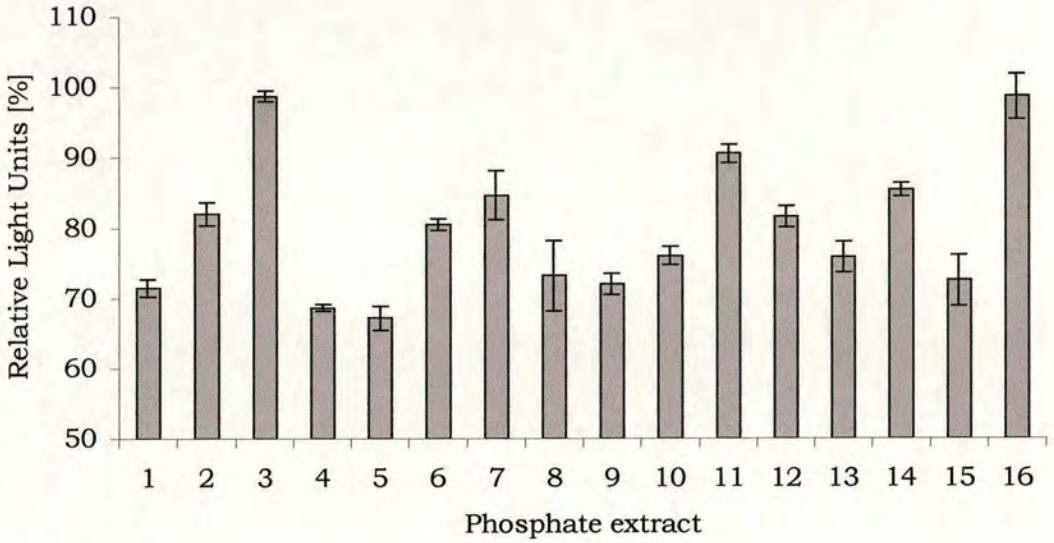


Fig. 4.19 Toxicity of phosphate extracts from Rutherglen Glencairn soil samples to *E. coli* pUCD607 compared with 0.02M phosphate control at pH 7.0.

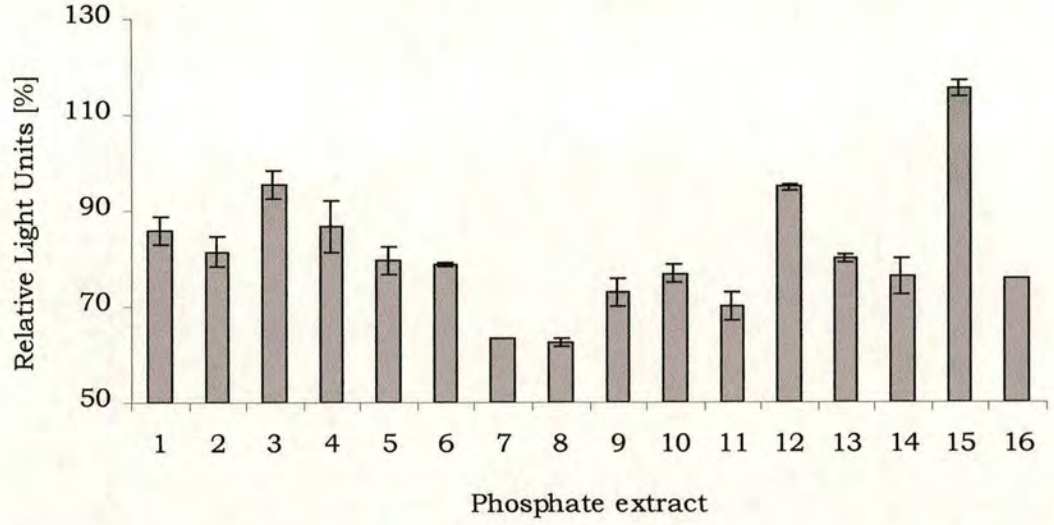


Fig. 4.20 Toxicity of phosphate extracts from Rutherglen Glencairn soil samples to *E. coli* pUCD607 compared with 0.02M phosphate controls at extract pH.

The toxicity of phosphate extracts resulting from the two sets of experiments (control pH 7.0 and own pH control) was plotted against Cr(VI) in phosphate extracts (Figure 4.21). It was observed that at Cr concentrations below 1mg/L, phosphate extracts from set 1 were less toxic than those from set 2. Between 1 and 2 mg/L Cr, the

toxicity of the two sets of experiments was similar. Above 2 mg/L Cr, the phosphate extracts compared to pH 7.0 proved more toxic than phosphate extracts compared to own pH controls. It was also observed that the toxicity of phosphate extracts from set 2 increased with increasing Cr(VI) concentration above 4 mg/L Cr(VI). This trend was less obvious in results from the set 1 experiments.

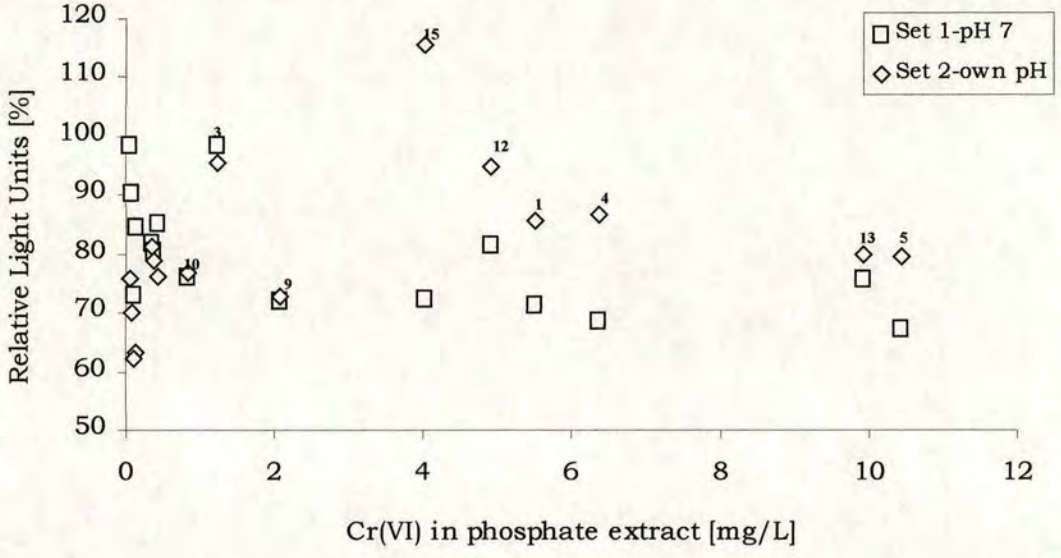


Fig. 4.21 Toxicity of phosphate extracts to *E. coli* pUCD607 for the two sets of experiments plotted against Cr(VI) in phosphate extracts.

The toxicity of phosphate extracts resulting from the two sets of experiments (control pH 7.0 and own pH control) was plotted against phosphate extracts pH (Figure 4.22). It was observed that below pH 7.5, the toxicity of phosphate extracts compared with a pH 7.0 control was either lower or similar to the toxicity of phosphate extracts compared with their own pH controls. At pH above 8.5 the toxicity of phosphate extracts in set 1 experiments was higher than that of set 2 experiments. Again the toxicity of set 2 experiments increased with the increase in pH.

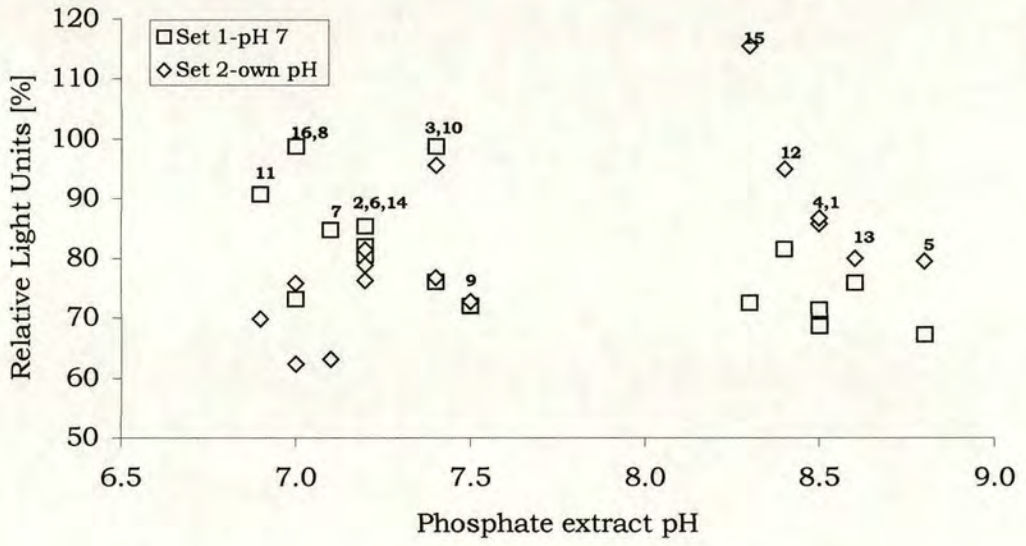


Fig. 4.22 Toxicity of phosphate extracts to *E. coli* pUCD607 for the two sets of experiments plotted against pH of phosphate extracts.

For concentrations above 4 mg/L Cr(VI) and above pH 8.3, the toxic effect was more obvious for phosphate extracts compared with own pH controls than those compared to the pH 7.0 control, with toxicity increasing with the increase in pH and Cr(VI). It remained difficult to separate the effect of pH from the effect of chemical composition on the toxicity of the phosphate extracts to *E. coli* pUCD607.

To explore the possible factors contributing to phosphate extract toxicity, a correlation matrix was calculated for the two sets of results (%RLU compared with control pH 7.0 and with own pH control).

For the experiments using pH 7.0 as control, significant correlations were found between toxicity (%RLU) and:

- Ca in soils (negative, r=-0.674, P=0.01)
- Fe (negative, r=-0.672, P=0.01)

- Cr in soils (negative, r=-0.636, P=0.01)
- Mg in soils (negative, r=-0.607, P=0.01).
- phosphate extracts pH (negative, r=-0.621, P=0.01)
- Cr(VI) in phosphate extracts (negative, r=-0.582, P=0.02)
- Cr in phosphate extracts (negative, r=-0.568, P=0.02)

Figures 4.23 and 4.24 show the relation between the possible factors mentioned above and the toxicity of phosphate extracts to *E. coli* pUCD607 (compared of phosphate extracts compared with pH 7.0 control).

For the experiments using phosphate solutions at their own pH as control (Figure 4.25), significant correlations were found between toxicity (%RLU) and:

- Si in phosphate extracts (positive, r=0.737, P=0.001)
- Cu in soils (negative, -0.623, P=0.01).
- phosphate extract pH (positive, r=0.547, P=0.02)

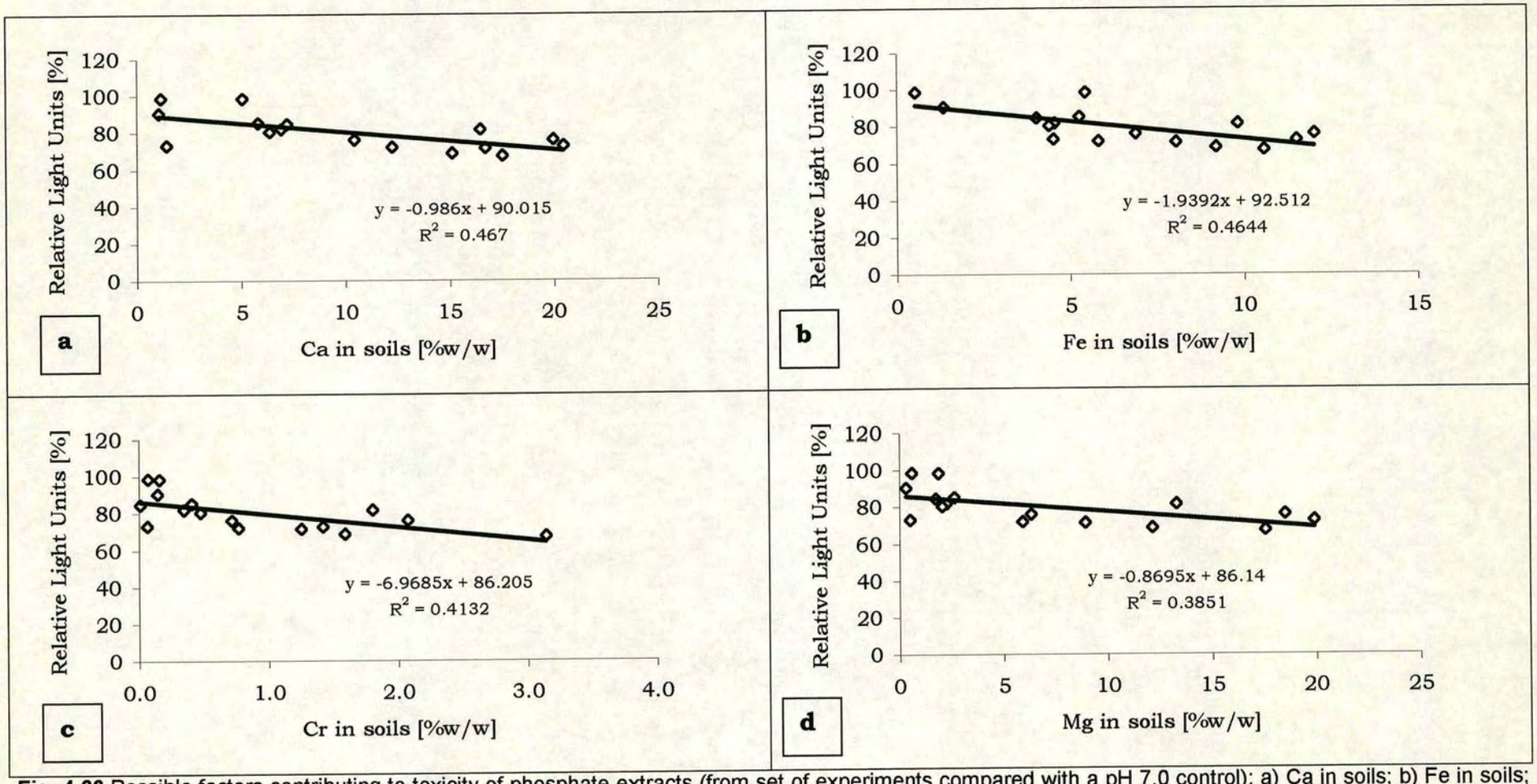
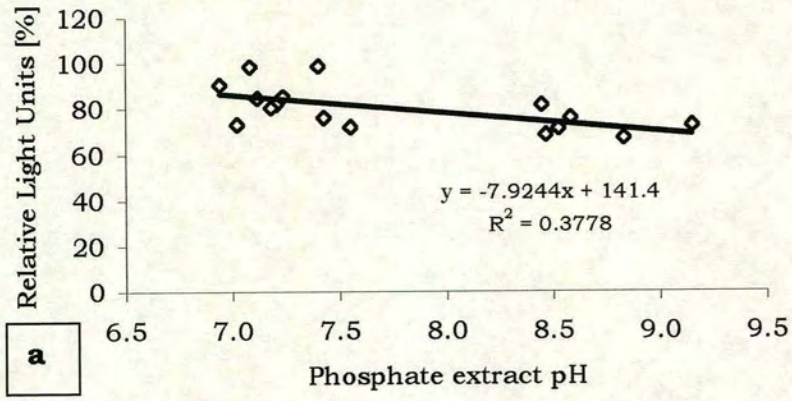
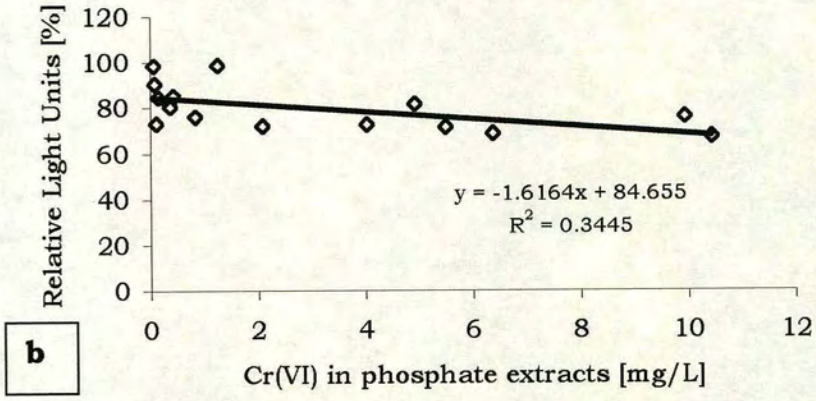


Fig. 4.23 Possible factors contributing to toxicity of phosphate extracts (from set of experiments compared with a pH 7.0 control): a) Ca in soils; b) Fe in soils; c) Cr in soils and d) Mg in soils.





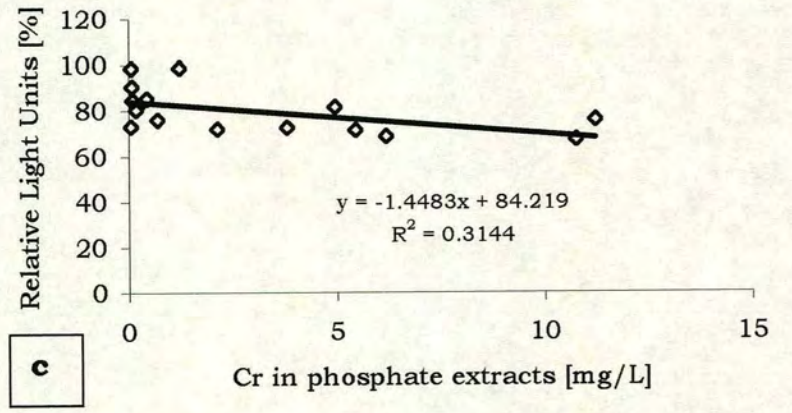


Fig. 4.24 Possible factors contributing to toxicity of phosphate extracts (from set of experiments compared with a pH 7.0 control): a) Phosphate extract pH; b) Cr(VI) in phosphate extracts and c) Total Cr in phosphate extracts.

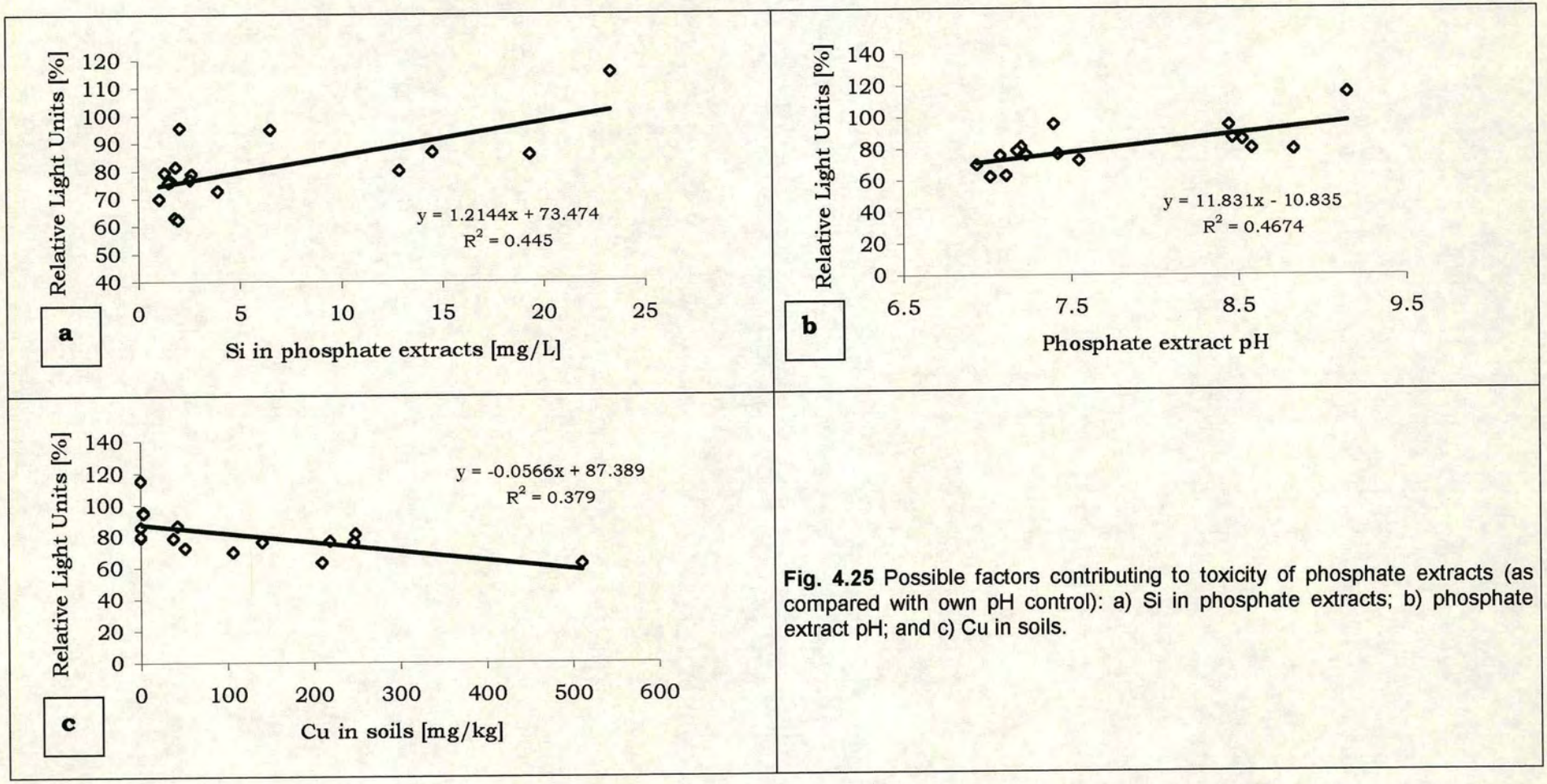


Fig. 4.25 Possible factors contributing to toxicity of phosphate extracts (as compared with own pH control): a) Si in phosphate extracts; b) phosphate extract pH; and c) Cu in soils.

4.9.2 Bioassays on soil pore waters

The toxicity of soil pore waters to *E. coli* pUC607 was not investigated, as in the eight out of thirteen soil pore waters extracted which were below pH 9.5, the chromium concentrations were low.

4.9.3 Bioassays on groundwater samples

Groundwater samples were stored for about 3 weeks before their toxicity was investigated using *E. coli* pUCD607 (Section 2.3.1). Sample BH402 was excluded from the experiments, as its pH exceeded pH 9.5. The pH of groundwater samples was measured again before the bioassays were performed. An increase of pH was observed compared to the pH at time of sampling (Figure 4.26). The pH measured at the time of the bioassays was used for comparison with %RLU.

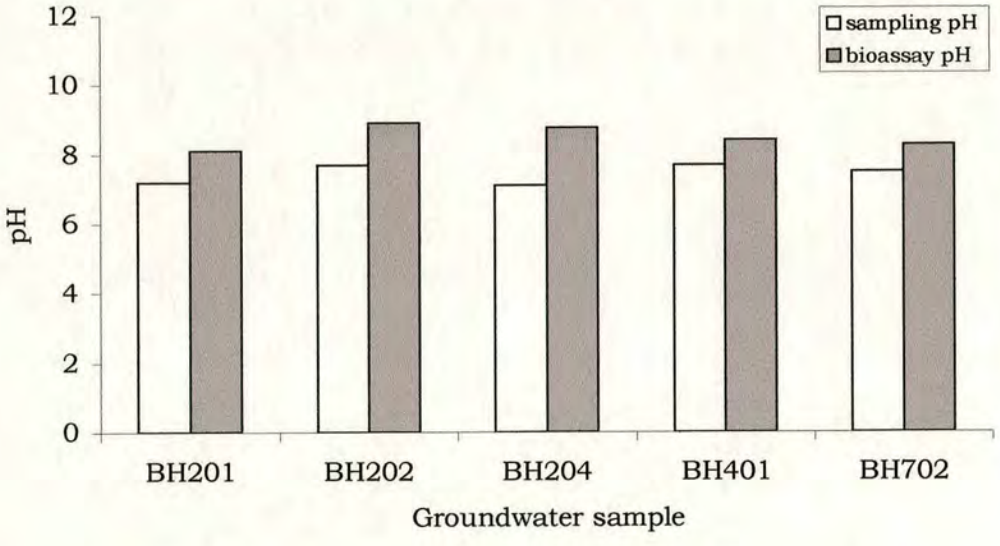


Fig. 4.26 Comparison of groundwater sample pH when sampled and during bioassay.

The toxicity of groundwater samples was compared to a pH 7.0 control and to the pH of individual groundwater samples. The toxicity of samples compared to the pH 7.0 control was higher than that of samples compared to controls at own pH (Figure 4.27).

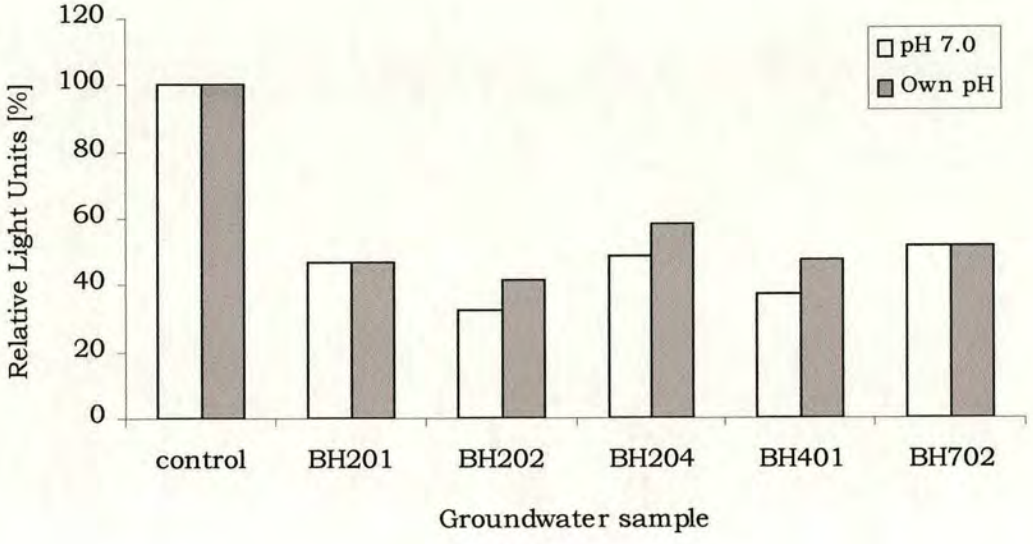


Fig. 4.27 Toxicity of groundwater samples compared to pH 7 control and own pH controls.

The highest toxicity (compared to a pH 7.0 control) was recorded for the groundwater sample BH202, followed by the sample BH401. A highly significant negative correlation was found between toxicity and groundwater pH ($r=-0.975$, $P=0.001$) (Figure 4.28), and a significantly positive correlation was found between toxicity and Ca ($r=0.895$, $P=0.01$) (Figure 4.29). However, these correlations have to be interpreted with care as the number of samples was too small, in statistical terms.

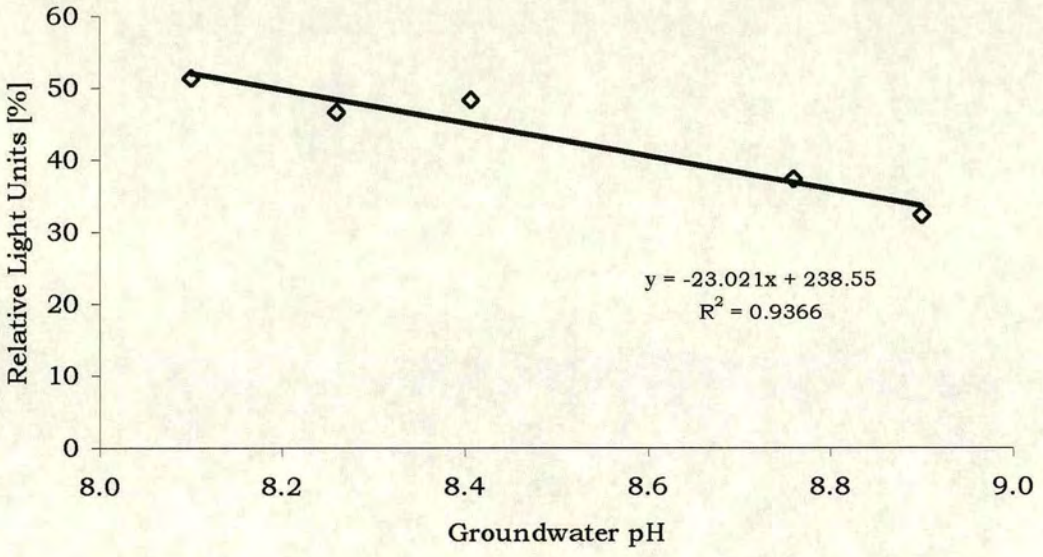


Fig. 4.28 Toxicity of five groundwater samples (compared with pH 7.0 control) against groundwater sample pH

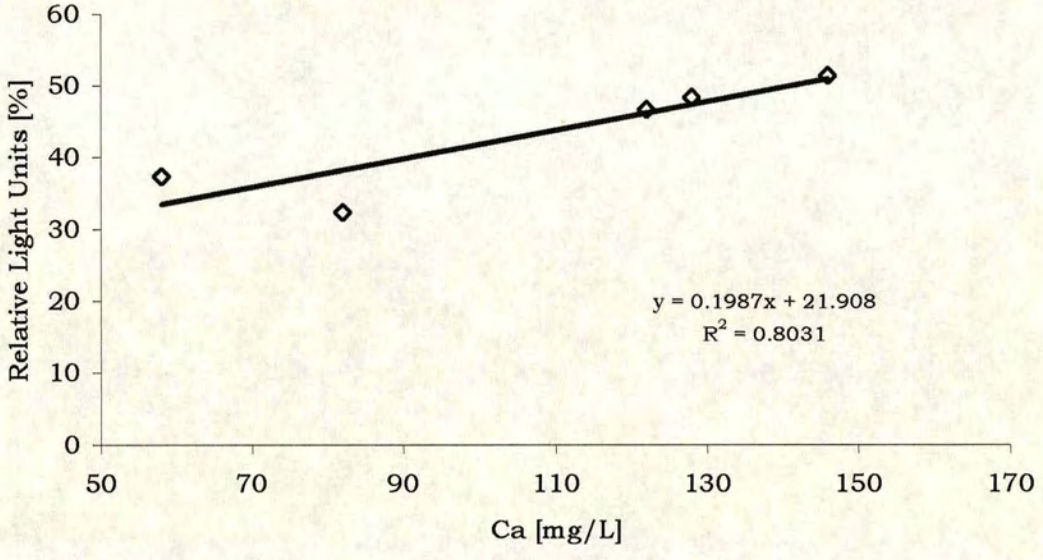


Figure 4.29 Toxicity of five groundwater samples (compared to pH 7.0) against groundwater Ca concentration.

4.9.4 Summary of results from bioassays on phosphate extracts and groundwater samples

- The toxicity of phosphate extracts and groundwater samples was different for the two sets of experiments used (control pH 7.0 and controls at individual extract pH).

- The effect of pH on toxicity was evident for both the phosphate extracts and groundwater samples, but it was more marked for the toxicity of groundwater samples.

- The effect of pH on toxicity of phosphate extracts was difficult to separate from the effect of chemical composition.

4.10 Discussion

The study of solid samples from the COPR-contaminated site under study (Rutherglen Glencairn Football Club, Site 4) showed that such material could not be characterised using the classification schemes for agricultural soils, but that it should be considered as urban soil, *i.e.* soil material having a non-agricultural use, being a man-made surface layer more than 50 cm thick, that has been produced by mixing, filling, or by contamination of land surface in urban and suburban areas (Bockheim, 1974). Throughout the text they are referred to as soil samples for ease.

The variability in physicochemical properties of the solid samples highlighted the complexity of urban soils in disposal sites (Bridges,

1987). It also gave the opportunity to compare soil samples with different chemical composition, pH and Cr contents.

Concentrations of Cr found in soil samples varied from 0 to 3.1% (w/w), depending on their COPR contents. According to the results from the alkaline digestion, just a small percentage of that Cr was found in the hexavalent form (0-0.4% w/w). Up to 0.07% (w/w) Cr(VI) was extracted by phosphate buffer. The statistical analyses suggested that the concentrations of Cr(VI) in phosphate extracts were correlated to pH and the concentrations of Ca, Mg and Fe in soil samples. The correlation with Ca, Mg and Fe is possibly related to the amounts of hydrocalumite mineral phase present in the soil samples. According to Thomas *et al.* (2001) hydrocalumite contains chromate as an exchangeable anion. Therefore, if the amount of hydrocalumite in some samples is higher than in others, then it could be expected that the amount of potentially available Cr would also be higher in those samples.

Soil pore waters obtained from the soil samples also showed a high variation in chemical composition, pH and Cr contents. Concentrations of Cr ranged from 0 to 43 mg/L and Cr was found entirely as Cr(VI).

Phosphate extracts and soil pore waters originating from soil samples 1, 4, 5, 12 and 13 had a high content of Cr(VI), although there was a difference in the relative amounts between soil pore waters and phosphate extracts, *i.e.* the highest concentrations of Cr(VI) in phosphate extracts were recorded for samples 5 and 13, while the highest concentrations of Cr(VI) in soil pore waters were recorded for samples 1 and 4. This could indicate that samples 5 and 13 contained more exchangeable Cr(VI) than samples 1 and 4.

The difference in composition between the soil pore waters and the phosphate extracts highlights some of the problems associated with the study of the toxicity of soil samples, the need to define how to study them and what to considered as the bioavailable fraction of contaminants in soils. Several studies have used operational speciation or fractionation to account for the forms in which contaminants are found in soils (Tessier *et al.*, 1979; Lake *et al.*, 1984; Beckett, 1989). The extraction would usually be designed to extract the element associated or bound to a particular soil phase, but in practice, the soil phase may be ill-defined and the extractant not specific to the phase. The use of soil pore waters to account for the bioavailable fraction of soil contaminants has been suggested, but in the case of contaminants such as Cr, which can be present in soils both in solution and as exchangeable anions in the solid phase, the use of soil pore waters to test the toxicity of soil samples might not be the best option. In this particular case, pore waters extracted might be indicators of the portion of Cr(VI) in solution, but might not be good indicators of other species which could potentially be made available, such as exchangeable forms of Cr(VI) and, therefore, toxicity could be underestimated.

In this study, the toxicity of soils and groundwater from COPR-contaminated sites was investigated using the biosensor *E. coli* pUCD607. Soil samples, phosphate extracts, soil pore waters and groundwater samples varied in their pH, with some having a pH greater than 9.5.

The experiments in Chapter 3 showed that the functionality of *E. coli* pUCD607 can be disturbed at high pH, and that in order to avoid additional stress from matrix pH, samples investigated should have a pH within the *E. coli* pUCD607 optimum pH range. Therefore, only solutions below pH 9.5 were tested.

The results obtained from the bioassays on the phosphate extracts in this chapter showed that they were more toxic than the solutions containing just Cr(VI) used in Chapter 3. EC_{25} values obtained in the experiments in Chapter 3 with synthetic chromium solutions showed that at pH between 8 and 9, a concentration of around 95 mg/L of chromium would be required to cause a reduction in luminescence of 25%. The highest concentration of chromium found in phosphate extracts was 11 mg/L, but the toxicity recorded was higher than that of synthetic solutions at the same pH, as concentrations as low as 2 mg/L of Cr(VI) in phosphate extracts resulted in more than 25% reduction in luminescence.

In groundwater samples it was again obvious that the toxicity was due to a combination of factors. EC_{50} values obtained for synthetic Cr(VI) solutions at pH 8.0 and 9.0 were 417 and 311 mg/L, respectively. The groundwater samples had concentrations of up to 30 mg/L but the luminescence detected was below 50% in all samples.

The difference in the toxicities of synthetic solutions, compared with solutions from environmental samples, indicates that a combination of factors, including contaminant concentration, pH, matrix chemical composition and the interactions between the contaminant(s) and various other chemical species present in the matrix, can influence the toxicity of environmental samples.

If chemical analysis alone had been compared with dose-response curves for synthetic solutions, the conclusions would have been that the solution-phases of the environmental samples tested were not significantly toxic, but it was actually found that they were much more toxic than expected.

4.11 Conclusions

The main findings of all the experiments reported in this chapter can be summarised, as follows:

- It is better to use the combination of biosensors and chemical analyses, as the toxicity of environmental samples is the result of chemical interactions between contaminants and environmental matrices and also depend on the organism exposed. *Escherichia coli* pUCD607 and chemical analysis can be used to measure the toxicity of environmental samples and, together, can suggest possible factors contributing to toxicity.

- This type of bioassay can give information on the toxicity due to a combination of factors, in this case Cr and pH effects, but cannot distinguish the toxicity caused by an individual contaminant. Unlike dedicated biosensors, such as those specific for As, Hg, or even Cr, the use of non-dedicated methods might be a better approach to study the toxicity of environmental samples.

- It was not possible to determine which was the major contributing factor to toxicity, since the pH effect could not be really separated from Cr(VI) concentration and/or chemical composition in general.

- The toxicity of real samples was much higher than expected, compared with the toxicity of synthetic solutions, indicating more complex interactions of chemical composition that determine the toxicity of samples.

- Toxicity depends on the bioavailability of a contaminant. This, in turn, is influenced by the contaminant's chemical form and physicochemical properties, the composition and characteristics of the surrounding medium and the possibility of uptake by, or contact between substance and organism. Therefore, including all possible contributing factors when studying toxicity might be more accurate than just referring to the toxicity of individual contaminants. In this sense the combination of a "general" biosensor such as *E. coli* pUCD607 with physicochemical analyses can give a better idea of the possible risks associated with contaminated sites.

Chapter 5

The Effect of Cr(VI) on Soil Microbial Communities, and Comparison with a Single Species Microbial Assay and a Plant Assay

5.1 Introduction

The experiments in Chapter 4 demonstrated that COPR-contaminated sites can have high levels of bioavailable Cr(VI) that are toxic to *E. coli* pUCD607. They also demonstrated that leaching

of COPR material can occur and that it could contaminate groundwater. In the same way, it could potentially reach healthy soils and affect organisms growing in them. Soil microbial communities are complex and can respond to contaminant loads in different ways (depending on the existence of detoxification or resistance mechanisms) and the response seems to be dependent on exposure route and time/dose relationships (Luoma, 1995).

Single species bioassays, such as the *E. coli* pUCD607 assay, measure the potential acute toxicity of the soil *ex situ*, but because the interaction of contaminants with other soil components and the different processes taking place in soils can have different effects on the toxicity of contaminants to soil microbial communities, this kind of assay might not reflect the effects of contaminants on indigenous soil microbial communities under chronic exposure conditions. Information on the chronic toxicity of contaminants on soil microbial communities and plant systems *in situ* is also important.

In order to better understand the toxicity of Cr(VI) in soils, a microcosm was devised in which acute and chronic effects were studied. Three soils with different Cr(VI) adsorption capacities and potentially different microbial communities were spiked with a series of increasing Cr(VI) concentrations and left to equilibrate for one month under controlled conditions. Physicochemical analysis and bioassays were used to study the toxicity of Cr(VI). Single species bioassays (*E. Coli* pUCD607 and a plant assay) were used to investigate the acute toxicity of Cr(VI) in soil solutions. The chronic effects of Cr(VI) on indigenous soil microbial communities were investigated by studying changes in the community structure, using signature lipid biomarkers (Section 1.2.4.2) and the metabolic capacity of such communities was studied by community level physiological profiles (Section 1.2.4.1).

5.2 Objectives and hypothesis

- assess the influence of Cr(VI) loads on the microbial community of soils with different adsorption capacity and/or land use;

- compare the acute and chronic toxicity of Cr(VI) in soils through different bioassays, *i.e.* single species bioassay, community assays and plant assays.

The hypothesis tested was that toxicity of Cr(VI)-contaminated soils to indigenous microorganisms could be predicted by the knowledge of the Cr(VI) adsorption capacity of the soil, which in turn depends on the pH, organic matter content and nature and concentration of mineral components.

5.3 Physicochemical properties of candidate soils

In order to select the soils for this experiment, the adsorption of Cr(VI), as chromate, was investigated in four Scottish soils that are among the most important and extensive agricultural soils in central and NE Scotland (Table 5.1).

The soils studied had different clay contents (6-26%) and physicochemical properties (Table 5.2, Figure 5.1). The soil samples were taken down to 30 cm depth during October/November 1999 as part of an independent study (Campbell *et al.*, 2001). Soil sub-samples were passed through a 6 mm stainless steel sieve, air-dried and stored. Most of the bulk samples remained stored at field

moisture, below 12°C, in double, thick polyethylene bags in a chamber subjected to natural light cycles and aeration.

Sub-samples of these soils were used both to test the Cr(VI) adsorption capacity (air-dried soil) and to set up the microcosm experiment (moist soil).

Table 5.1 Classification of soils for which Cr adsorption was measured.

Site	Major soil subgroup	Soil Association	Soil Series	Land use/cover
ALDROUGHTY	Humus-iron podzol (cultivated)	Boyndie	Boyndie	Arable (barley stubble undersown with grass)
GLENCORSE	Noncalcareous gley (imperfect)	Rowanhill	Winton	Improved pasture
ARNHALL	Alluvial soil	Alluvium	Essil	Rough grassland
HARTWOOD N. RESERVOIR	Noncalcareous gley (poor)	Rowanhill	Rowanhill	Permanent pasture

Table 5.2 Physicochemical properties of soils for which Cr adsorption was measured (taken from Campbell *et al.*, 2001)

Site						pH		H	%INT	%BSTC	%INT	%BSTC	%
	Bible N°	%N	%C	C:N ratio	%LOI	H_2O	$CaCl_2$	meq/100g	Sand	Sand	Silt	Silt	Clay
ALDROUGHTY	647810	0.08	1.22	15.9	3.44	6.94	6.56	1.18	66.21	44.0	15.7	37.8	18.1
ARNHALL	647812	0.25	3.26	13.0	8.07	5.87	5.32	4.23	80.13	65.4	13.8	28.5	6.08
GLENCORSE	647811	0.18	2.58	14.2	7.84	5.74	5.38	4.71	55.58	39.3	18.3	34.7	26.1
HRT - N. RESERVOIR	647820	0.31	5.00	16.1	12.3	5.56	5.02	8.36	56.40	33.9	23.5	46.0	20.1

Site		Elemental composition (mg/kg)							Exchangeable Cations (mg/kg)				Oxalate Extractables (mg/kg)			
	Bible N°	Cd	Cr	Cu	Ni	P	Pb	Zn	Ca	Na	K	Mg	Al	Fe	Mn	Si
ALDROUGHTY	647810	<0.5	7.83	8.66	4.06	610	12.1	47.7	7.78	0.05	0.66	3.80	966	2042	173.1	348
ARNHALL	647812	<0.5	26.0	7.59	14.7	360	40.2	53.5	8.64	0.13	0.14	2.58	1500	3597	39.3	648
GLENCORSE	647811	<0.5	40.6	21.2	27.8	681	70.3	101	10.1	0.08	0.14	2.12	1751	6655	297	339
HRT - N. RESERVOIR	647820	<0.5	43.6	10.0	11.6	773	39.1	47.8	8.83	0.11	0.22	1.83	3478	11680	596	560

5.4 Study of soil Cr(VI) adsorption capacity

The adsorption of Cr(VI) was measured at standard conditions, 25°C and 1 atm. Three hypotheses were tested when investigating the Cr(VI) adsorption on these soils:

- Soils with higher concentrations of Al and Fe oxides would have a higher Cr(VI) adsorption capacity.
- Organic matter content would have an effect on the adsorption of Cr(VI).
- The adsorption of Cr(VI) onto soils would depend on soil pH.

One gram of air-dried soil (< 6 mm) was weighed into 50 ml centrifuge tubes. One ml of a K_2CrO_4 solution (twelve different concentrations) and 25 ml of 0.1M KNO_3 were added. Tubes were equilibrated by shaking for 24 hours at constant temperature. Duplicates were used for each dilution.

After equilibration, the pH of each suspension was measured. The tubes were immediately centrifuged at 4500 rpm for 10 minutes and the supernatant of each tube was collected and filtered through a 0.33 μm filter. The filtered solutions were analysed for Cr(VI) and total Cr (Section 2.2.5 and 2.2.6).

The experimental adsorption isotherms (Figure 5.2) were prepared by plotting the amount of chromate anion (CrO_4^{2-}) adsorbed per soil unit, q (mmol CrO_4^{2-}/kg soil), when in equilibrium with a concentration of Cr(VI) in solution, c (mmol CrO_4^{2-}/L). The highest adsorption capacities were observed for Hartwood-NR (Soil series Rowanhill) and Glencorse (Soil series Winton), followed by Arnhall

(Soil series Essil). The lowest chromate adsorption was observed in Aldroughty (Soil series Boyndie).

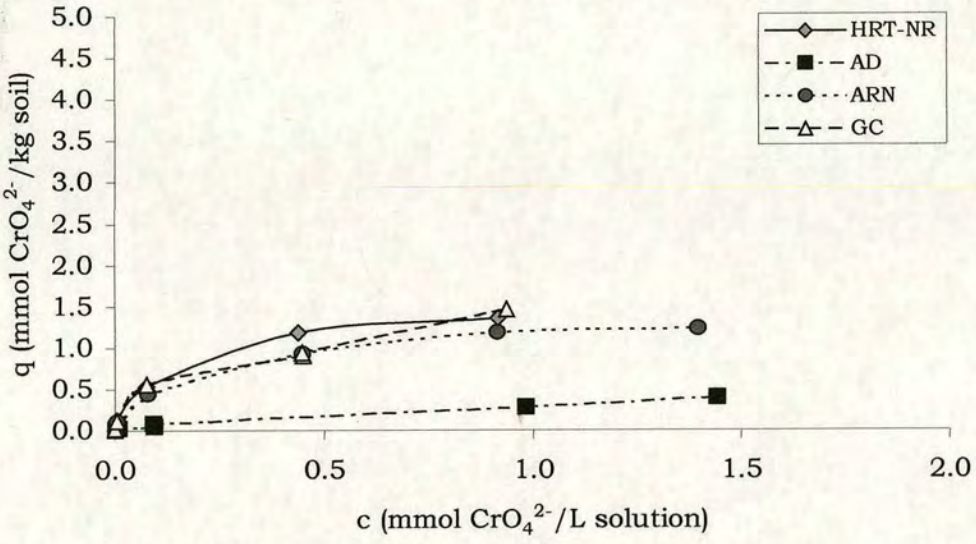


Fig. 5.2 Experimental adsorption isotherms for Hartwood-NR (HRT-NR), Aldroughty (AD), Arnhall (ARN), Glencorse (GC).

The two-surface Langmuir equation was used to model the adsorption of chromate in the soils studies (details of how they were calculated are included in Appendix 1). Other authors have previously used this equation to model the adsorption of chromate (Zachara *et al.*, 1989) and phosphate (Holford and Wedderburn, 1978) in soils.

Models for Cr(VI) sorption for each soil were plotted in Figure 5.3 and compared with the experimental data. Aldroughty and Arnhall were better represented by the models than Hartwood-NR and Glencorse.

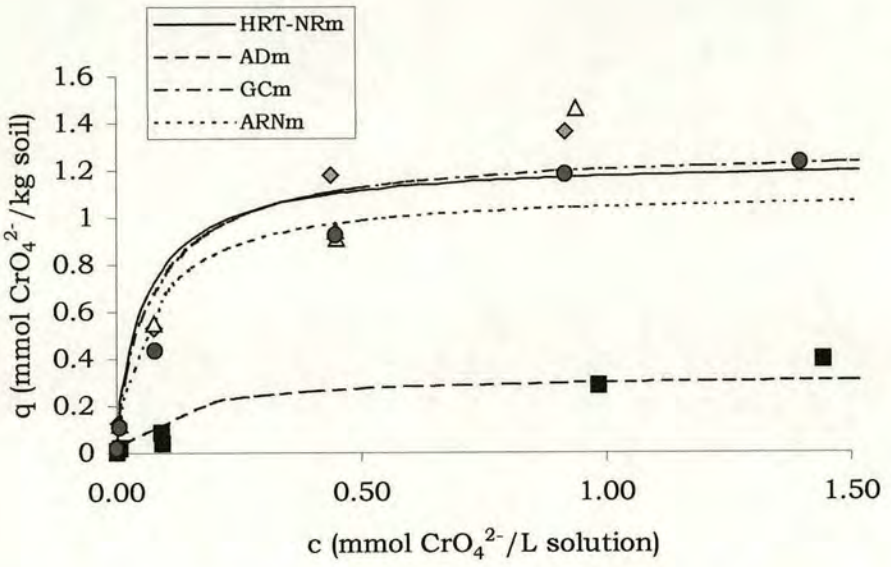


Fig. 5.3 Adsorption isotherms using experimental and modelled data for: Hartwood ◇; Glencorse △; Arnhall ●; Aldroughty ■

The sorption of Cr(VI) in Aldroughty (q_{max}= 0.33 mmol CrO_4^{2-}/kg soil), was the lowest, followed by Arnhall (q_{max}= 1.19 mmolCrO_4^{2-}/kg soil). Glencorse and Hartwood showed the highest adsorption capacies, with q_{max} of 1.29 and 1.24, respectively.

According to the experimental and modelled data, the soil pH and iron oxide contents might be the main contributing factor to soil Cr(VI) adsorption. A higher pH resulted in lower chromate adsorption. Chromium (VI) adsorption experiments using activated carbon (data not included here) showed that when activated carbon was rinsed with an acid solution, the adsorption of Cr(VI) was higher than when using non-acid-rinsed carbon. Other studies have reported similar results. Ainsworth *et al.* (1989) found that at low solution concentrations, chromate adsorption on goethite samples increased sharply from 0 to almost 100% as pH decreased from 10 to 7.

A higher iron oxide content resulted in a higher Cr(VI) adsorption. The relationship with %C was not obvious, although this was a small set of soil samples from which to draw conclusions. Zachara *et al.* (1989) reported that chromate adsorption in subsurface soil horizons was greatest in lower pH materials enriched with kaolinite and crystalline iron oxides.

The three soils selected for the experiment were Aldroughty, Arnhall and Glencorse, in order to have soils with different adsorption capacity and two soils with similar adsorption capacity, but a potentially different microbial community structure (due to land cover/use).

5.5 Microcosm set-up

Twelve 400 g sub-samples of each soil were spiked with solutions with increasing Cr(VI) concentrations (as K_2CrO_4) and their moisture content adjusted to 50% of their Water Holding Capacity (WHC) (calculations are included in Appendix 2). In order to try to discern possible effects of Cr from pH effects, reference controls were used, whereby sodium hydroxide solutions (no chromium), at the same pH as the chromium solutions used, were added to soils.

The Cr(VI) concentrations spiked in the soils were selected so they would cover a range that would include the EC_{50} values observed in the *E. coli* pUCD607 bioassays performed with synthetic solutions (Chapter 3). Concentrations of Cr(VI) expected in the soil solutions ranged from 0.1 to 50 mmol/L (5.2 to 2600 mg/L) (Appendix 2).
Soils were stored in 2.5 L Kilner Jars (the size of the jar allowed for head space to provide enough oxygen for microorganism respiration)

and covered with Parafilm™ (to allow gas exchange with the atmosphere). The jars were kept under darkness at 15°C and 90% humidity. The soils were left to equilibrate for 30 days, weighing the jars regularly to detect and correct moisture losses.

5.6 Physicochemical analyses

After 30 days of incubation, soil sub-samples were collected and analysed or extracted as follows:

5.6.1 Soil pH

The addition of increasing concentrations of Cr(VI) to soils had a different effect on soil pH in the different soils. Of the three soils, Aldroughty had the least variation, *i.e.* a better buffering capacity, with the pH ranging between 7.3 and 7.6 (mean 7.4). The range of pH measured in Arnhall samples was 6.0 to 6.5 (mean 6.3) and that for Glencorse was 5.6 to 6.4 (mean 6.1). In order to visualise the effect of the addition of Cr(VI) on soil pH, the difference between the final and original pH (soil without Cr(VI)) was plotted *versus* the amount of Cr(VI) added (Figure 5.4).

5.6.2 Soil pore water extraction and analysis

The soil pore water was extracted as described in Section 2.2.1, using approximately 150 g of soil. To avoid the alteration of the physicochemical properties of the soil pore water, samples were extracted without modifying the water holding capacity of the soil sub-samples (*i.e.* at 50% WHC). For some soils the extraction was

carried out twice, as not enough solution was obtained from a single extraction.

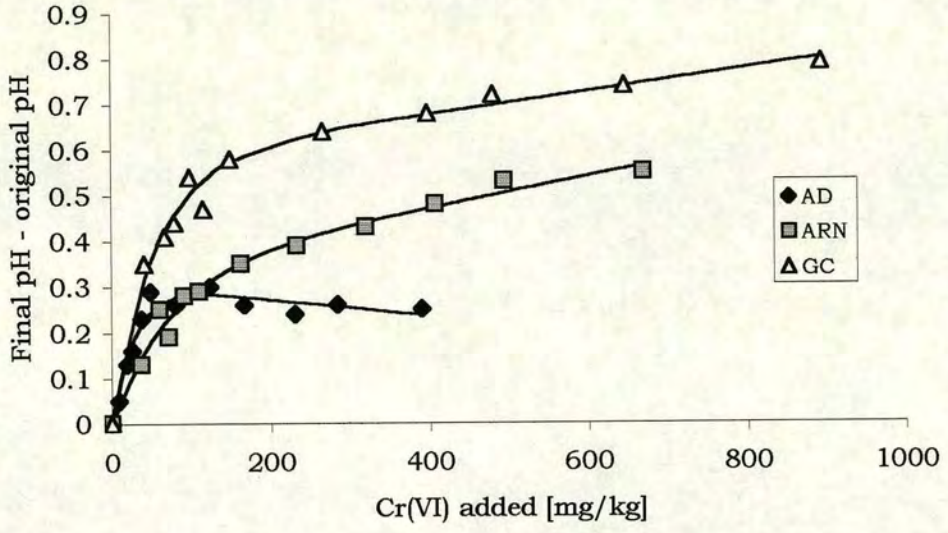


Fig. 5.4 The effect of Cr(VI) added on soil pH – the difference between final and original pH (soil without Cr(VI)). The continuous line represents the fitted values obtained by regression (exponential plus linear trend). Aldroughty (AD), Arnhall (ARN), Glencorse (GC).

The pH values recorded for Arnhall (4.1-6.0) and Glencorse (4.0-5.9) soil pore waters were considerably lower than those recorded for the soils from which they were extracted and a high variation was observed (Figure 5.5). The pH values of Aldroughty soil pore waters (6.8-7.3) were less variable and were more similar to the soil pH, again indicating a good buffering capacity in this soil.

The composition of the soil pore waters was analysed for total dissolved Cr and other metals by ICP (Tables 5.3 to 5.5) and for Cr(VI) by the colorimetric method described in Section 2.2.5.

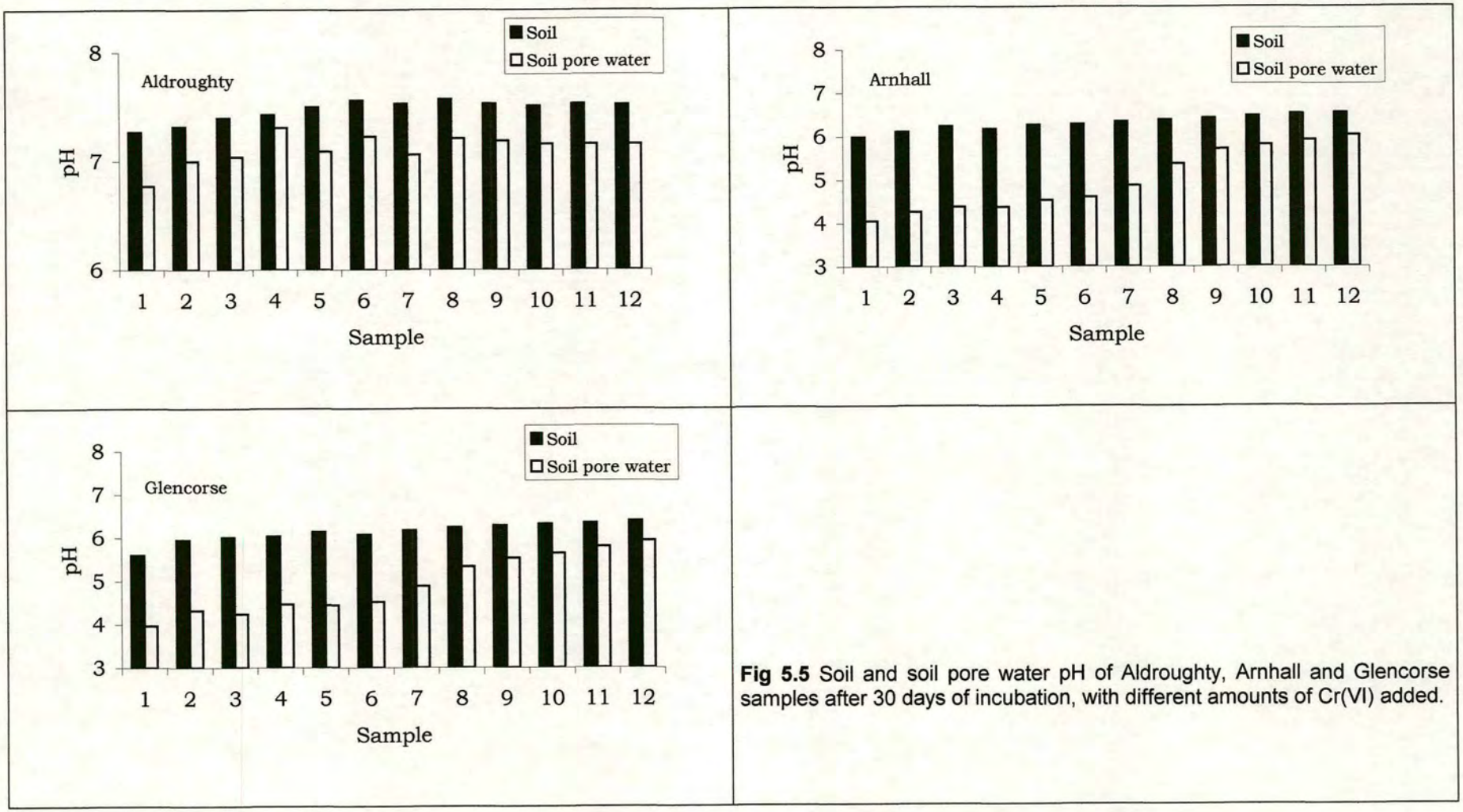


Fig 5.5 Soil and soil pore water pH of Aldroughty, Arnhall and Glencorse samples after 30 days of incubation, with different amounts of Cr(VI) added.

Table 5.3 Chemical compositions of the pore waters extracted from Aldroughty.

Jar	pH	Al [mg/L]	Ca [mg/L]	Cr [mg/L]	Fe [mg/L]	Mg [mg/L]	Mn [mg/L]	Na [mg/L]	P [mg/L]	S [mg/L]	Si [mg/L]	Ti [mg/L]	V [mg/L]	Zn [mg/L]
1	6.8	0.367	168	0.543	0.233	76.1	0.010	30.2	2.68	26.6	9.80	0.138	0.058	2.03
2	7.0	0.391	174	3.26	0.124	76.5	0.029	26.3	1.63	26.3	10.1	0.074	0.059	2.38
3	7.0	0.522	157	21.3	0.251	70.5	0.020	25.6	3.21	27.1	9.17	0.064	0.050	2.31
4	7.3	0.257	156	40.9	0.093	71.0	0.011	25.9	2.03	27.6	9.28	0.094	0.035	2.35
5	7.1	0.696	150	73.0	0.369	68.7	0.029	26.5	2.46	26.5	9.28	0.137	0.046	2.57
6	7.2	0.252	158	109	0.117	71.4	0.010	26.0	3.64	27.2	8.88	0.151	0.085	1.78
7	7.1	0.456	179	228	0.342	81.6	0.017	28.1	3.32	28.2	9.28	0.235	0.123	2.58
8	7.2	0.737	218	397	0.501	93.3	0.021	28.8	2.82	28.9	8.28	0.289	0.140	2.65
9	7.2	1.19	244	554	1.09	104	0.056	29.3	3.07	28.1	7.66	0.307	0.168	3.12
10	7.2	0.910	282	791	0.609	116	0.012	30.5	2.31	28.6	5.94	0.368	0.248	3.16
11	7.2	6.15	316	1011	5.39	128	0.211	31.6	1.49	28.9	14.9	0.858	0.315	3.22
12	7.2	0.984	366	1391	0.894	143	0.007	27.8	3.04	27.9	4.17	0.621	0.457	2.24
13	6.2	0.449	166	0.023	0.355	72.7	0.037	29.5	0.946	26.8	9.20	0.145	0.049	2.27
14	6.9	0.771	174	0.023	0.696	77.5	0.073	33.2	1.96	27.6	10.5	0.099	0.032	2.27
15	6.9	1.65	166	0.022	1.61	75.3	0.106	34.2	2.76	26.8	12.6	0.179	0.039	1.61
Mean	7.0	1.05	205	-	0.845	88.4	0.043	28.9	2.49	27.5	9.27	0.251	0.127	2.44
SD	0.27	1.46	67.0	-	1.32	23.5	0.054	2.69	0.76	0.87	2.48	0.22	0.12	0.47
SE	0.070	0.377	17.3	-	0.342	6.08	0.014	0.695	0.196	0.225	0.640	0.057	0.032	0.121

Table 5.4 Chemical compositions of the soil pore waters extracted from Arnhall.

Jar	*pH*	*Al* [mg/L]	*Ca* [mg/L]	*Cr* [mg/L]	*Fe* [mg/L]	*Mg* [mg/L]	*Mn* [mg/L]	*Na* [mg/L]	*P* [mg/L]	*S* [mg/L]	*Si* [mg/L]	*Ti* [mg/L]	*V* [mg/L]	*Zn* [mg/L]
1	4.05	0.369	197	0.001	0.153	48.7	0.021	29.2	0.306	10.4	7.62	0.074	0.045	2.18
2	4.26	0.265	187	2.57	0.109	46.2	0.016	29.9	3.17	13.1	8.58	0.124	0.001	2.58
3	4.38	0.209	164	8.31	0.172	42.0	0.014	25.5	0.386	14.1	7.95	0.076	0.032	1.11
4	4.37	0.281	161	6.25	0.107	40.6	0.014	27.1	1.33	13.1	7.87	0.124	0.053	2.22
5	4.53	0.210	160	12.5	1.95	40.7	0.018	21.9	0.629	13.3	7.13	0.021	0.001	0.44
6	4.6	0.291	157	15.5	0.094	40.9	0.014	24.1	1.10	14.0	7.01	0.021	0.001	0.47
7	4.87	0.285	150	28.1	0.221	39.2	0.011	21.8	0.949	12.9	7.03	0.031	0.052	0.36
8	5.37	0.438	153	58.8	0.362	39.7	0.018	28.6	0.001	14.0	8.64	0.111	0.073	2.40
9	5.71	1.003	160	128	2.31	41.3	0.037	25.1	1.75	15.1	8.85	0.139	0.049	1.51
10	5.81	0.533	168	215	0.385	43.5	0.011	29.9	0.868	17.3	8.77	0.174	0.089	2.21
11	5.92	0.292	175	320	0.639	45.9	0.012	32.5	0.781	18.2	7.96	0.236	0.132	2.49
12	6.03	0.427	212	561	0.468	55.7	0.002	31.7	1.90	20.0	7.91	0.302	0.159	2.56
Mean	4.99	0.38	170	-	0.58	43.7	0.02	27.3	1.10	14.6	7.94	0.12	0.06	1.71
SD	0.73	0.22	19.09	-	0.75	4.80	0.01	3.62	0.86	2.66	0.67	0.09	0.05	0.89
SE	0.21	0.06	5.51	-	0.22	1.39	0.00	1.04	0.25	0.77	0.19	0.02	0.01	0.26

Table 5.5 Chemical compositions of the soil pore waters extracted from Glencorse.

Jar	pH	Al [mg/L]	Ca [mg/L]	Cr [mg/L]	Fe [mg/L]	Mg [mg/L]	Mn [mg/L]	Na [mg/L]	P [mg/L]	S [mg/L]	Si [mg/L]	Ti [mg/L]	V [mg/L]	Zn [mg/L]
1	3.97	0.484	144	0.067	0.129	30.6	0.028	22.7	0.147	3.72	9.42	0.128	0.001	2.64
2	4.32	0.396	130	2.63	0.051	29.0	0.018	23.9	0.001	6.40	8.63	0.091	0.041	1.82
3	4.24	0.521	121	7.01	0.070	27.0	0.023	24.6	0.740	7.51	9.29	0.102	0.032	2.31
4	4.46	0.367	128	9.67	0.039	29.1	0.019	23.9	0.001	7.25	8.12	0.106	0.057	1.94
5	4.44	0.448	135	13.7	0.036	31.1	0.024	27.4	2.21	9.55	8.36	0.098	0.033	2.49
6	4.52	1.564	132	19.3	1.16	29.5	0.054	24.7	0.865	9.68	10.2	0.134	0.047	2.39
7	4.88	0.847	129	44.6	0.448	28.9	0.028	25.9	0.954	11.0	9.75	0.157	0.032	3.00
8	5.33	0.308	150	125	0.110	33.7	0.021	27.0	0.545	13.4	8.14	0.158	0.035	2.65
9	5.53	0.338	177	244	0.222	40.5	0.019	28.5	0.626	14.8	8.78	0.204	0.068	2.52
10	5.64	0.513	198	323	0.385	44.6	0.019	27.9	1.68	13.2	7.99	0.203	0.142	2.13
11	5.8	2.36	266	638	2.23	60.7	0.050	33.7	2.06	16.3	9.23	0.450	0.190	3.46
12	5.93	1.19	380	1121	0.956	82.2	0.014	36.5	2.47	17.4	6.07	0.547	0.301	3.94
13	4.08	1.04	151	0.001	0.693	31.6	0.056	27.9	1.67	3.77	9.09	0.116	0.049	2.43
14	4.09	0.385	150	0.001	0.121	31.8	0.047	24.9	2.12	3.67	9.60	0.083	0.033	2.75
15	4.44	0.947	160	0.001	0.752	34.0	0.062	35.7	1.79	4.22	10.9	0.117	0.033	2.35
Mean	4.78	0.781	170	-	0.49	37.6	0.03	27.7	1.19	9.46	8.90	0.18	0.07	2.59
SD	0.68	0.57	68.7	-	0.60	15.04	0.02	4.33	0.85	4.74	1.12	0.14	0.08	0.55
SE	0.18	0.15	17.7	-	0.16	3.88	0.00	1.12	0.22	1.22	0.29	0.04	0.02	0.14

Figure 5.6 shows the difference between Cr expected in soil pore waters (calculated using the double-surface Langmuir equation) and the experimental values, as measured by ICP and the diphenylcarbazide colorimetric method. This shows that, in general, the adsorption models used underestimated the adsorption capacities of the three soils.

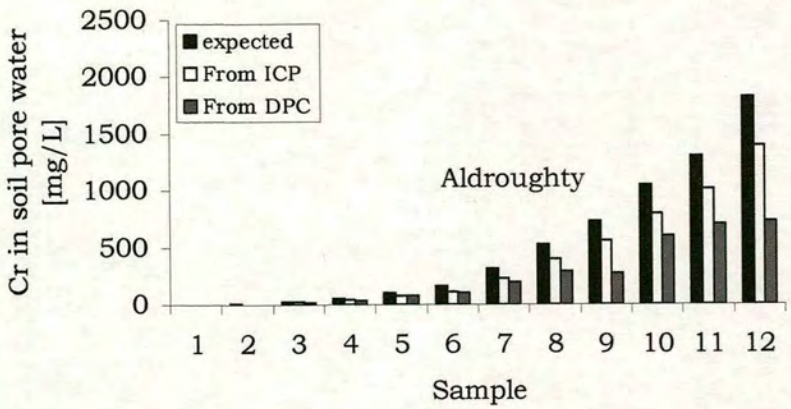
The difference between expected and found Cr in soil pore waters was:

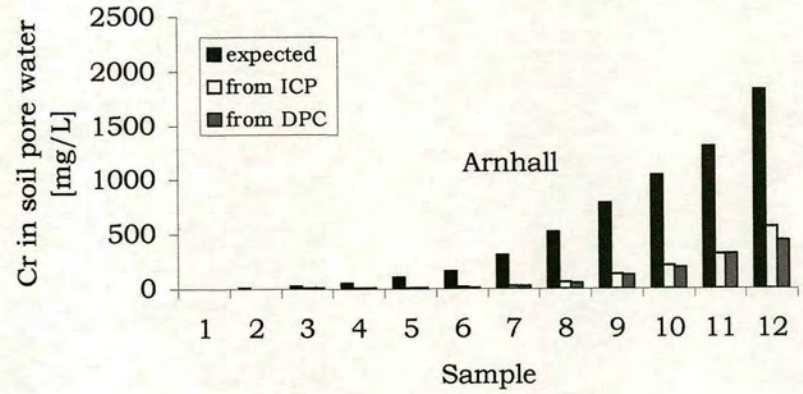
- 18-37% (mean=26%, SD=5%) for Aldroughty soil pore waters;
- 51-90% (mean=79%, SD=13%) for Arnhall soil pore waters;
- 49-88% (mean=74%, SD=13%) for Glencorse soil pore waters.

The underestimation of the Cr adsorption capacity in Aldroughty was not as high as that for Arnhall and Glencorse. The adsorption experiments shown previously, suggested that Aldroughty data gave the best fit with the Langmuir equation and that the fits for Arnhall and Glencorse were poor.

Possible factors contributing to the underestimation of the Cr adsorption capacity of these soils include:

Soil/solution ratio. The adsorption isotherm experiments were carried out with the solution in excess, *i.e.* 1 g of soil in 26ml of solution, while the microcosm samples were carried out with soil in excess, 1g of soil contained between 0.17 to 0.24 g of water.





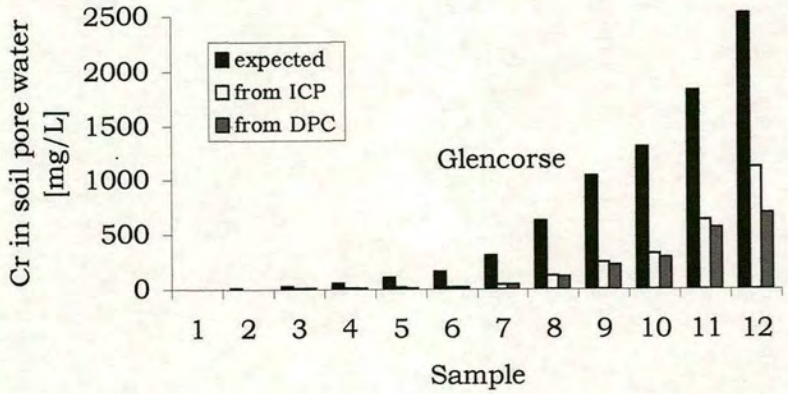


Fig. 5.6 Comparison of expected (calculated) Cr(VI) in soil pore waters and Cr(VI) and total Cr found by experimental methods.

- *Equilibration time.* The equilibration time normally used in adsorption experiments was 24 hours, whereas the samples in the microcosm experiment were equilibrated for 30 days, which could have allowed further adsorption or reduction/precipitation reactions in presence of organic matter.

- *Adsorption equation.* The Langmuir equation is a simple model that does not take into account the mechanism of adsorption. The adsorption of Cr(VI) onto soil surfaces is clearly pH dependent and models that include the protonation of surfaces might represent Cr adsorption better. It is noted that the model represented Aldroughty better, with an original pH around 7.0, while the models for the two other more acidic soils were poor. More complex models (Zachara *et al.*, 1989) might explain Cr(VI) adsorption better, although in this experiment a simpler approach was required, as it was more important to distinguish the relative Cr(VI) adsorption capacity of the soils to design the experiment.

- *Experimental error.* Possibly associated with the soil pore water extraction procedure or analysis.

The chemical analysis of soil pore waters also showed that the values obtained for Cr(VI) were lower (by approximately 35%) than those obtained for total Cr by ICP in all the samples. This fact excludes the possible interference of sample turbidity, as the turbidity increases the absorbance measured.

The difference between total Cr and Cr(VI) might possibly have been due to:

- *Cr complexation with organic matter.* When extracting soil solutions by the centrifugation method, solutions were filtered through a No.40 Whatman filter paper. Normally solutions were clear and no further filtration was used. According to the manufacturer, the pore size of this filter paper allows particles below 2.5 μm to pass through. If Cr was complexed by organic matter when analysed by the colorimetric method, the absorbance measured would be lower than expected, as it is the free Cr(VI) that reacts with diphenylcarbazide. When analysed by ICP, the samples were acidified with 2% HNO_3, potentially releasing the complexed Cr.

- *Cr(VI) reduction to Cr(III).* Another possibility could have been the reduction of part of the Cr(VI) in the most acidic soils by organic matter or Fe(II) (Tokunaga *et al.*, 2001). Chromium (III) has a very low solubility in water and is normally found precipitated in solid phases. Therefore, if any Cr(III) was present it would have been associated with particles passing through the filter.

- *Experimental error.* Due to sample manipulation or spectrophotometer calibration (standards).

5.6.2.1 Effect of Cr additions to soil on the chemical composition of the soil pore waters

The addition of increasing concentrations of Cr(VI) to soils also influenced the levels of other elements found in soil pore waters:

- *Calcium.* The accumulated analysis of variance obtained from linear regression analysis showed a clear effect of Cr on Ca concentrations in soil pore waters in Aldroughty and Glencorse ($P<0.001$), but there was not a strong effect in Arnhall ($P=0.245$). Calcium concentrations in the pore waters of Aldroughty and Glencorse samples increased with the increase in Cr added to the soil (Figure 5.7).

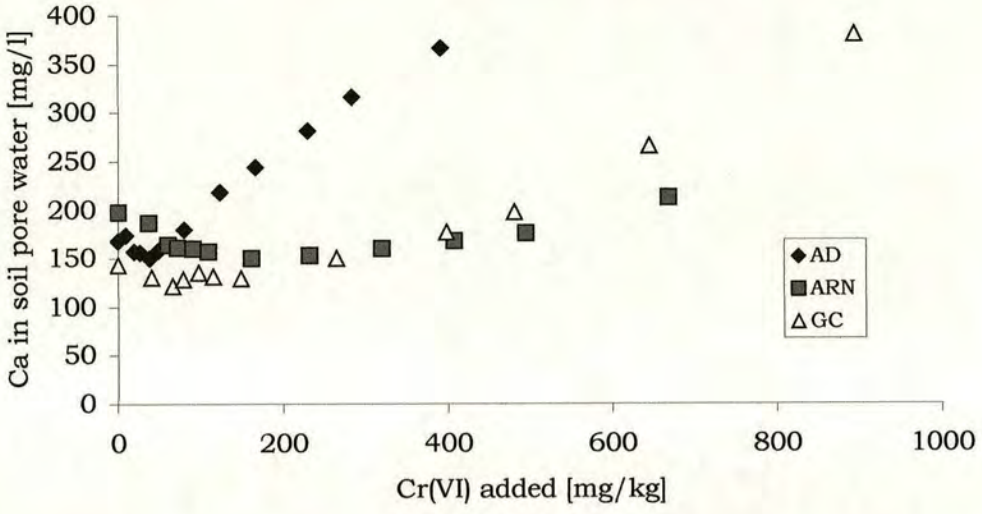


Fig. 5.7 The effect of Cr(VI) added to soils on the Ca concentration in soil pore waters.

- *Magnesium.* The effect of the addition of Cr(VI) to soils on the Mg concentrations in soil pore water (Figure 5.8) was significant in Aldroughty ($P<0.001$) and Glencorse ($P<0.001$), but in the latter the change in concentrations was not as large as that for Aldroughty. In Arnhall, Mg concentrations increased slightly with the addition of Cr and was only very weakly significant ($P=0.079$).

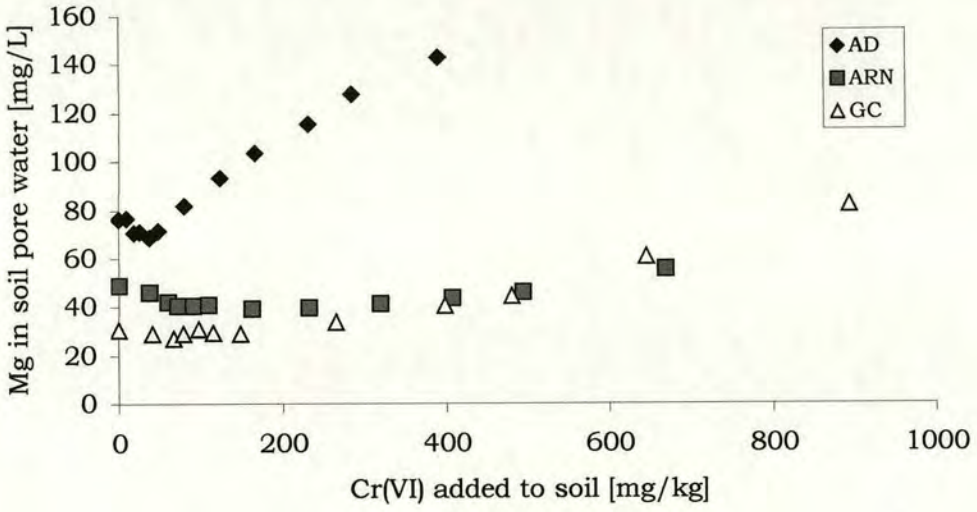


Fig. 5.8 The effect of Cr(VI) added to soils on the Mg concentration in soil pore waters.

- *Sulphur.* Aldroughty had the highest concentrations of S in soil pore waters. Chromium treatment and type of soil had a significant effect on the concentration of S in soil pore waters (0.045) (Figure 5.9). The difference in S concentrations in the soil pore waters from the three soils was highly significant (P<0.001).

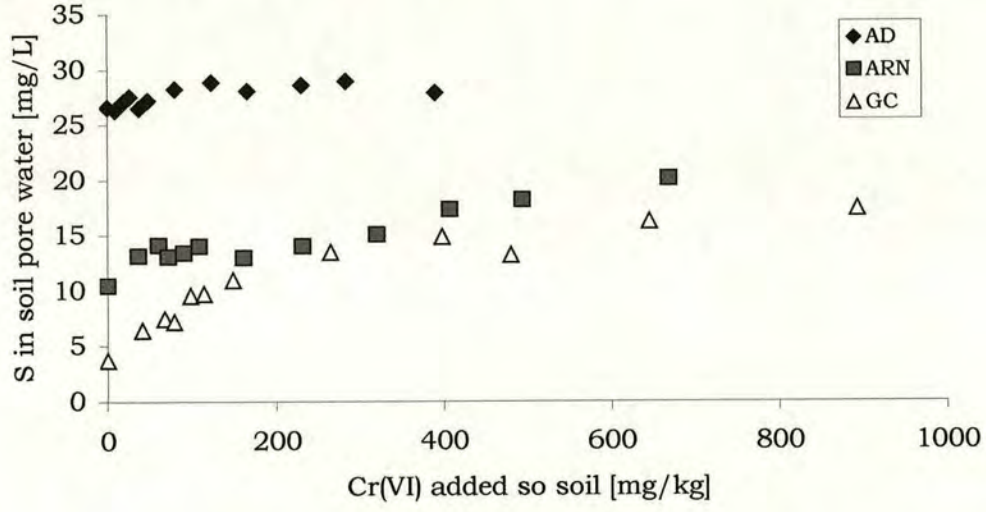


Fig. 5.9 The effect of Cr addition to soils on the S concentration in soil pore waters.

- Other elements. The concentrations of other elements in soil pore water (*i.e.* Al, Fe, Mn, Na, P, Si, Ti, V, Zn) did not seem to be particularly affected by the addition of Cr(VI) to soils.

5.6.2.2 Possible factors contributing to soil pore water pH

A correlation matrix using the chemical components of soil pore water suggested that sulphur contributed significantly ($P<0.001$) to soil pore water pH (Figure 5.10). The highest contribution was observed for Glencorse followed by Arnhall. There was no significant correlation found for Aldroughty. Although not shown in the correlation matrix, Cr(VI) added to soil was also highly correlated with the soil pore water pH (Figure 5.11).

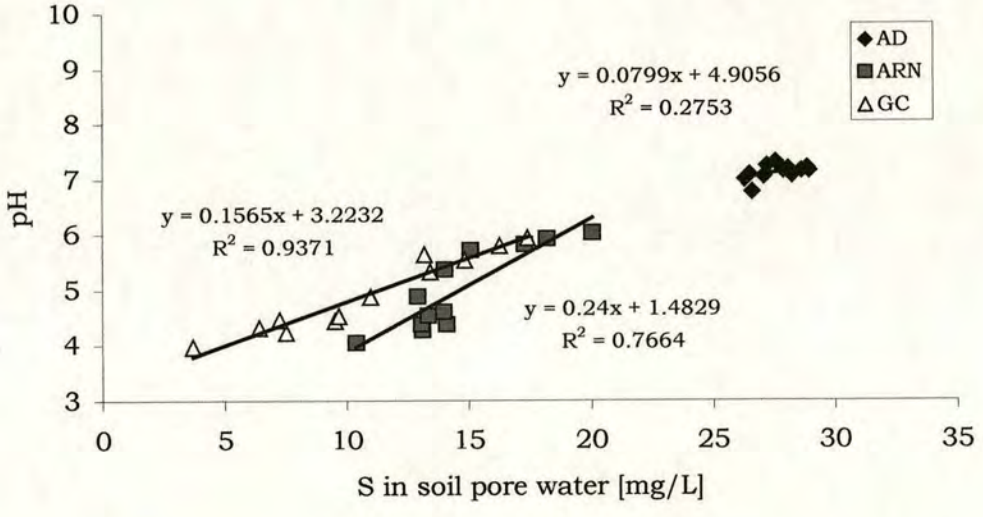


Fig. 5.10 Correlation of S concentration with soil pore water pH.

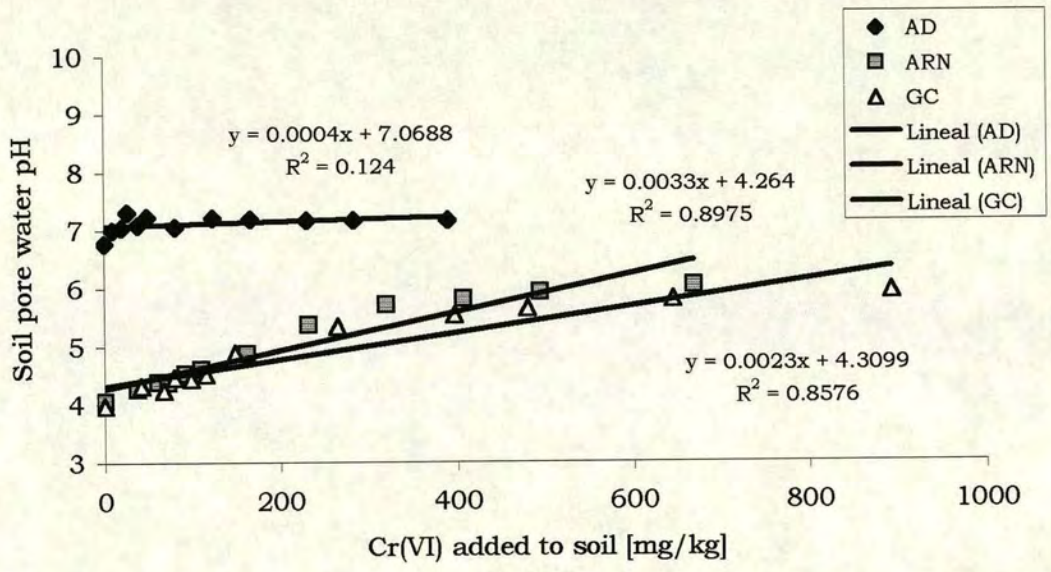


Fig. 5.11 Correlation of amount of Cr(VI) added with soil pore water pH.

5.6.3 Phosphate extracts

Phosphate extractions were carried out in all samples and analysed for Cr(VI) but levels obtained were very low (data not shown) and they were not used for further analyses.

5.6.4 Soil digestions

Soils were digested (Section 2.2.2) and analysed by ICP-OES (Section 2.2.6) with the purpose of checking the concentrations of Cr, as it was expected that concentrations of the other elements would not change. Chromium concentrations found (data not shown) were the result of the Cr(VI) added plus the concentrations already in the soils (Table 5.2).

5.7 Biological analyses

The potential (eco)toxicity of Cr(VI) added to soils was studied using a series of biological analyses including single species bioassay (*E. coli* pUCD607, root development of barley plants) and community bioassays (changes in community structure and metabolic capacity). Results for each analysis were compared with Cr(VI) added and the chemical composition of soil pore waters. Possible factors contributing to (eco)toxicity were studied. The assays were also compared with each other.

The development of new techniques that do not rely on microbial culturing now make it possible to study indigenous soil microbial communities. While there are many possible methods, two approaches were used to measure both the taxonomic and functional diversity of the communities: biological markers (phospholipid fatty acid (PLFA) analysis) to study the community structure; and a metabolic approach, based on community-level physiological profiles (CLPP) which uses the addition of carbon sources to determine the community functionality.

5.7.1 Analysis of soil pore waters using bioassays

The soil pore waters obtained were analysed using the biosensor *E. coli* pUCD607, as described in Section 2.3.1.

Each soil was compared with the experimental control (*i.e.* soil to which no Cr(VI) had been added) and with a pH control which represented the average of the soil pore water pH values (reference pH control).

5.7.1.1 Bioassays in soil pore waters from Cr-contaminated Aldroughty soils

The response of *E. coli* pUCD607 to soil pore waters obtained from Aldroughty Cr-contaminated soils was compared with a reference pH 7.0 control (phosphate buffer) and to its experiment control. A similar negative exponential response was observed for both Cr and Cr(VI) in soil pore waters (Figure 5.12).

The response of *E. Coli* pUCD607 (using pH 7.0 as control) compared to Cr(VI) added (Figure 5.13) showed a similar trend.

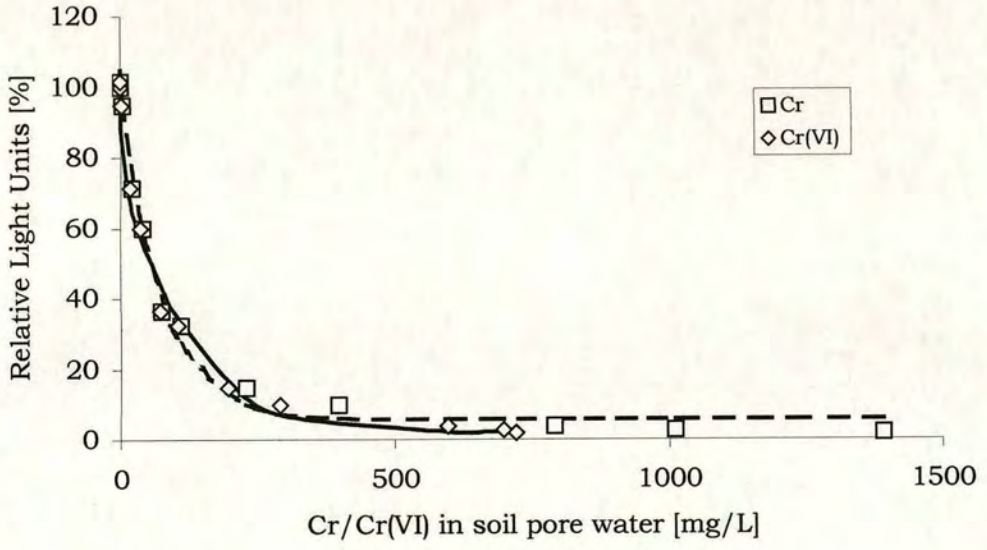


Fig. 5.12 The effect of Cr and Cr(VI) on luminescence of *E. coli* pUCD607 in soil pore waters obtained from Cr-contaminated Aldroughty soils, relative to a pH 7.0 solution control (100%).

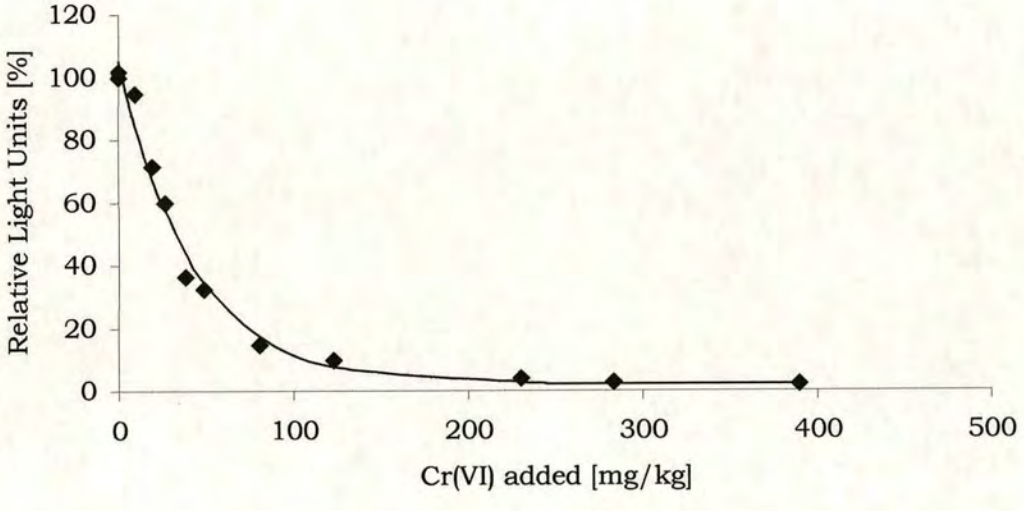


Fig 5.13 The effect of Cr(VI) added to soil on the luminescence of *E. coli* pUCD607 in Cr-contaminated Aldroughty soils, relative to a pH 7.0 solution control (100%).

The luminescence of bacteria resulting from assays which used their experiment control (100% RLU) also showed there was no significant

difference (P=0.899) between the response to Cr and Cr(VI) in soil pore waters (Figure 5.14). A similar trend was observed when luminescence was plotted against Cr(VI) added to soil (Figure 5.15).

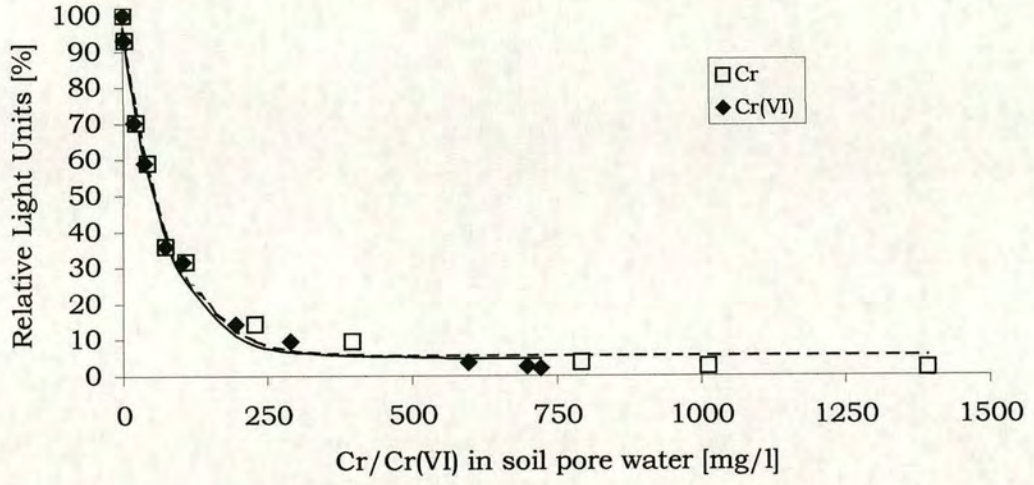


Fig 5.14 The effect of Cr and Cr(VI) on the luminescence of *E. coli* pUCD607 in soil pore waters obtained from Cr-contaminated Aldroughty soils. The control for the assay was the soil pore water with no Cr (*i.e.* microcosm control).

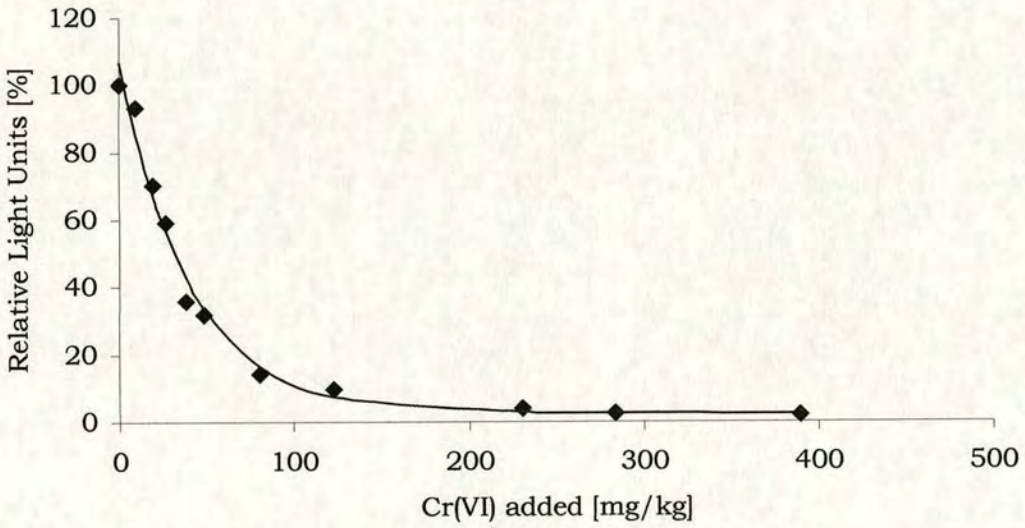


Fig 5.15 The effect of Cr(VI) added to soils on the luminescence of *E. coli* pUCD607 in for soil pore waters obtained from Cr-contaminated Aldroughty soils. The control for the assay was the soil pore water with no Cr (*i.e.* microcosm control).

EC values (Table 5.6) for all curves in Aldroughty were obtained by using the parameters from the regression analysis.

Table 5.6 EC values for the toxicity of Cr to *E. coli* pUCD607 - Aldroughty

Control	Compared with Cr	Cr species	EC_{50}*	EC_{25}*
Experiment	In soil pore water	Cr(VI)	52	19
Reference pH 7.0	In soil pore water	Cr(VI)	53	21
Experiment	In soil pore water	Cr_{tot}	54	21
Reference pH 7.0	In soil pore water	Cr_{tot}	56	22
Experiment	Added	Cr(VI)	31	14
Reference pH 7.0	Added	Cr(VI)	32	14

*Units for EC values compared with Cr added are in mg/kg of soil and mg/L Cr in soil pore water.

From table 5.6 it was observed that:

- there was no significant difference in %RLU using either a the reference pH 7.0 control or its experiment control. The pH of the experiment control in this case was 6.8 and the reference pH control used was 7.0;

- there was no significant difference between the toxicity measured caused by Cr(VI) and total Cr from soil pore waters, which might indicate that all the Cr present in the sample was Cr(VI).

5.7.1.2 Bioassays in soil pore waters from Cr-contaminated Arnhall soils

The response of *E. coli* pUCD607 to soil pore waters obtained from Arnhall Cr-contaminated soils was compared with a reference pH 4.9 control (phosphate buffer) and to its experiment control. Figure 5.16

shows the response of bacteria to both Cr and Cr(VI) in soil pore water. A similar negative exponential response was observed for both.

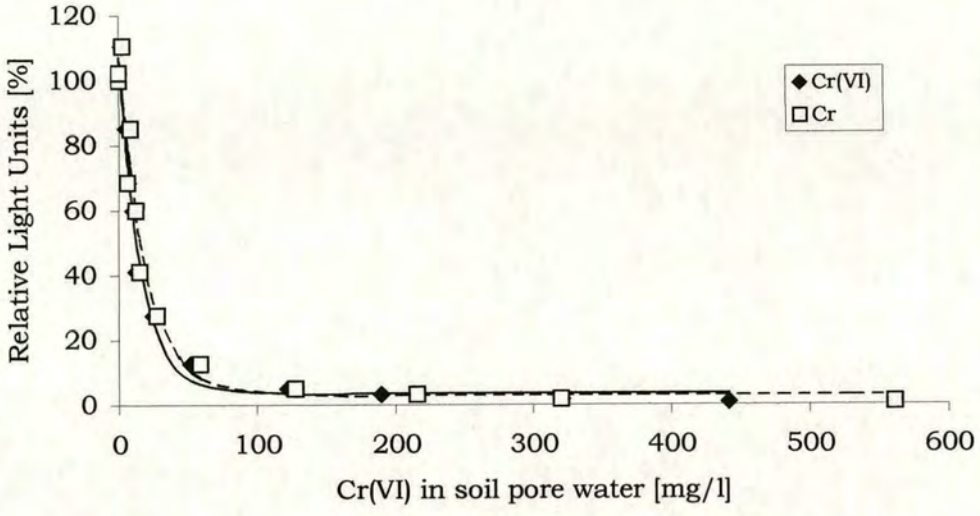


Fig. 5.16 The effect of Cr and Cr(VI) on the luminescence of *E. coli* pUCD607 in soil pore waters obtained from Cr-contaminated Arnhall soils, relative to a pH 4.9 solution control (100%).

The response of *E. coli* pUCD607 (using the reference pH 4.9 as control) compared to Cr added (Figure 5.17) also showed a negative exponential response, but the slope was less pronounced than for Cr(VI) and Cr in soil pore water.

Once again there was no significant difference between the response to Cr and Cr(VI) in soil pore water for Arnhall soil pore waters when compared with their experiment control (Figure 5.18). When luminescence was plotted against Cr added to the soil (Figure 5.19) however, there was in this case, a less pronounced negative exponential response.

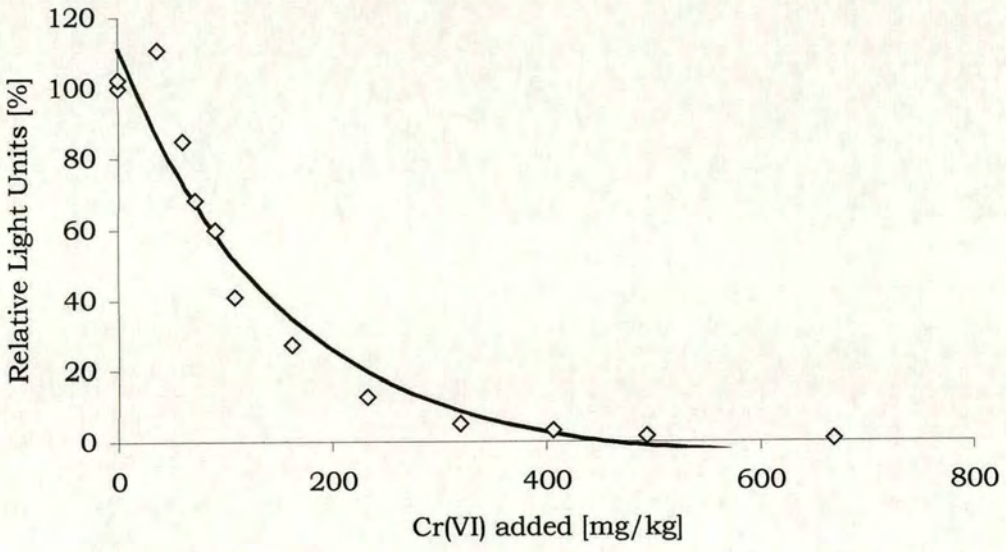


Fig 5.17 The effect of Cr(VI) added to soil on the luminescence of *E. coli* pUCD607 in soil pore waters obtained from Cr-contaminated Arnhall soils, relative to a pH 4.9 solution control (100%).

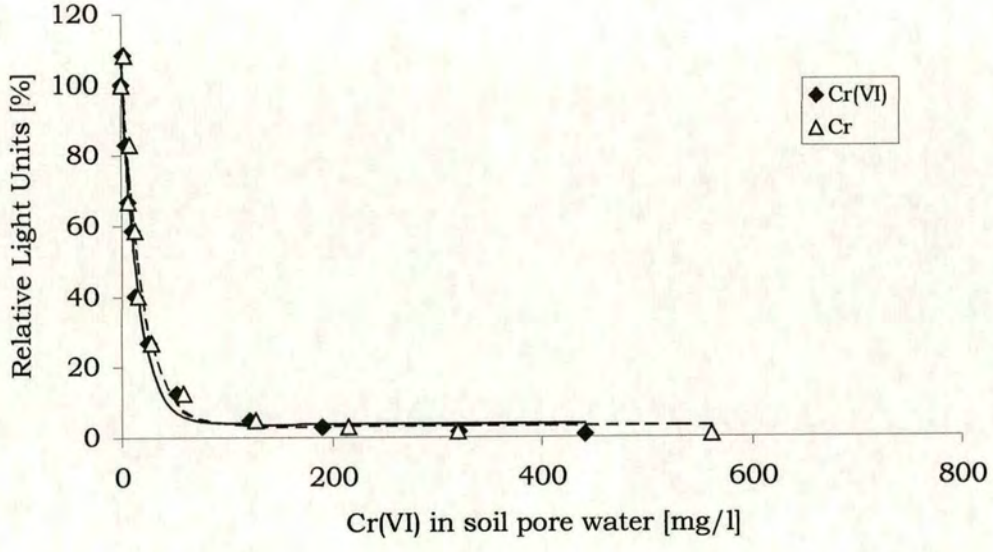


Fig. 5.18 The effect of Cr and Cr(VI) on the luminescence of *E. coli* pUCD607 in soil pore waters obtained from Cr-contaminated Arnhall soils. The control for the assay was the soil pore water with no Cr (*i.e.* microcosm control).

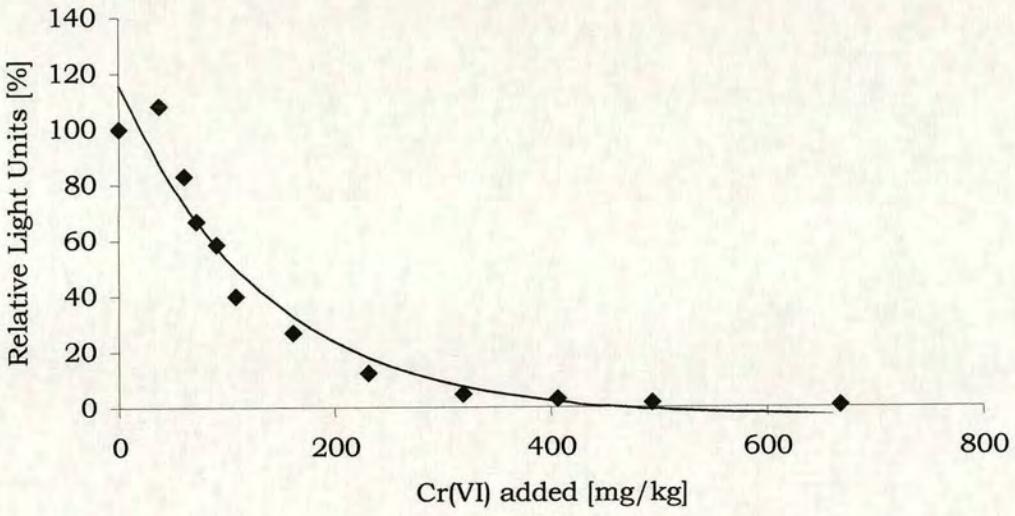


Fig. 5.19 The effect of Cr(VI) added to soils on the luminescence of *E. coli* pUCD607 in soil pore water obtained from Cr-contaminated Arnhall soils. The control for the assay was the soil pore water with no Cr (*i.e.* microcosm control).

EC values (Table 5.7) for all curves in Arnhall were obtained by using the parameters from regression analysis.

Table 5.7 EC values for the toxicity of Cr to *E. coli* pUCD607- Arnhall.

Control	Compared to Cr	Cr species	EC_{50}*	EC_{25}*
Experiment	In soil pore water	Cr(VI)	12	6
Reference pH 4.9	In soil pore water	Cr(VI)	13	6
Experiment	In soil pore water	Tot Cr	15	7
Reference pH 4.9	In soil pore water	Tot Cr	16	7
Experiment	Added	Cr(VI)	109	57
Reference pH 4.9	Added	Cr(VI)	113	56

*Units for EC values compared with Cr added are in mg/kg of soil and mg/L Cr in soil pore water.

From table 5.7 it was observed that:

- there was no significant difference between the toxicity induced by Cr(VI) and total Cr from soil pore waters;

- there was no significant difference in %RLU using either the reference pH 4.9 control or its experiment control. The pH of the experiment control in this case was 4.0, and the average pH used for control was 4.9.

5.7.1.3 Bioassays in soil pore waters from Cr-contaminated Glencorse soils

The response of *E. coli* pUCD607 to soil pore waters obtained from Glencorse Cr-contaminated soils was compared with a reference pH 4.9 control (phosphate buffer) and to its experiment control.

A similar negative exponential response was observed for both Cr and Cr(VI) in soil pore water using the reference pH 4.9 control and there was no significant difference between them (Figure 5.20). Figure 5.21 shows the response of bacterial luminescence (compared with the reference pH 4.9 control) compared with Cr(VI) added. Again, a negative exponential concentration-response curve was obtained.

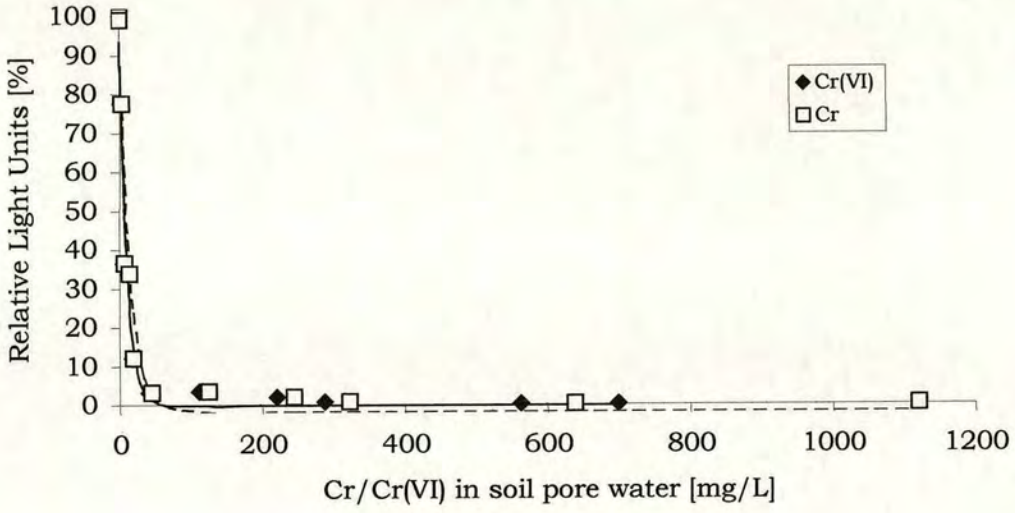


Fig. 5.20 The effect of Cr and Cr(VI) on the luminescence of *E. coli* pUCD607 in soil pore waters obtained from Cr-contaminated Glencorse soils, relative to a pH 4.9 solution control (100%).

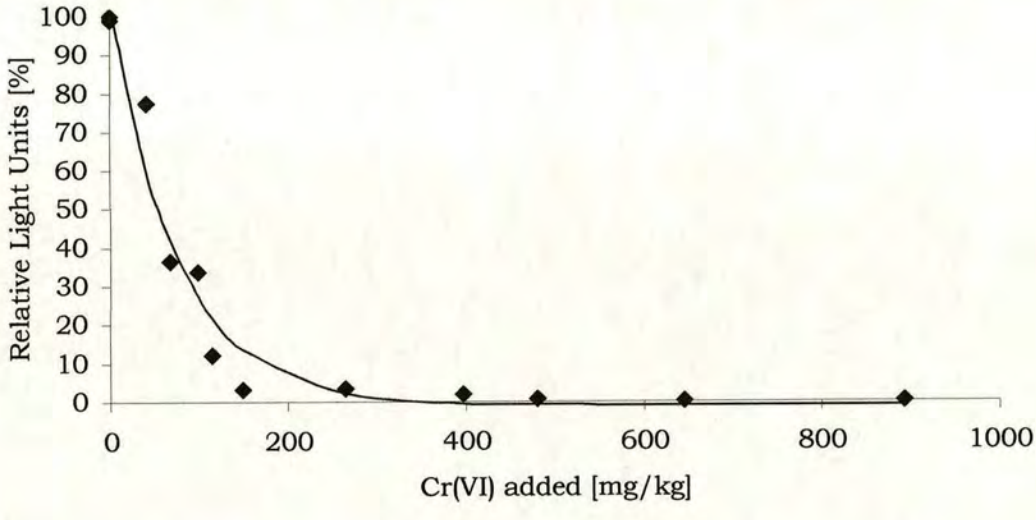


Fig. 5.21 The effect of Cr(VI) added on the luminescence of *E. coli* pUCD607 in soil pore waters obtained from Cr-contaminated Glencorse soils, relative to a pH 4.9 solution control (100%).

In Figure 5.22 the luminescence of bacteria resulting from assays with Glencorse soil pore waters (compared with their experiment control) was plotted *versus* Cr and Cr(VI). Again there was no

significant difference between the response to Cr and Cr(VI) in soil pore waters. When luminescence was plotted against Cr(VI) added to soil (Figure 5.23), a less pronounced negative exponential curve was obtained.

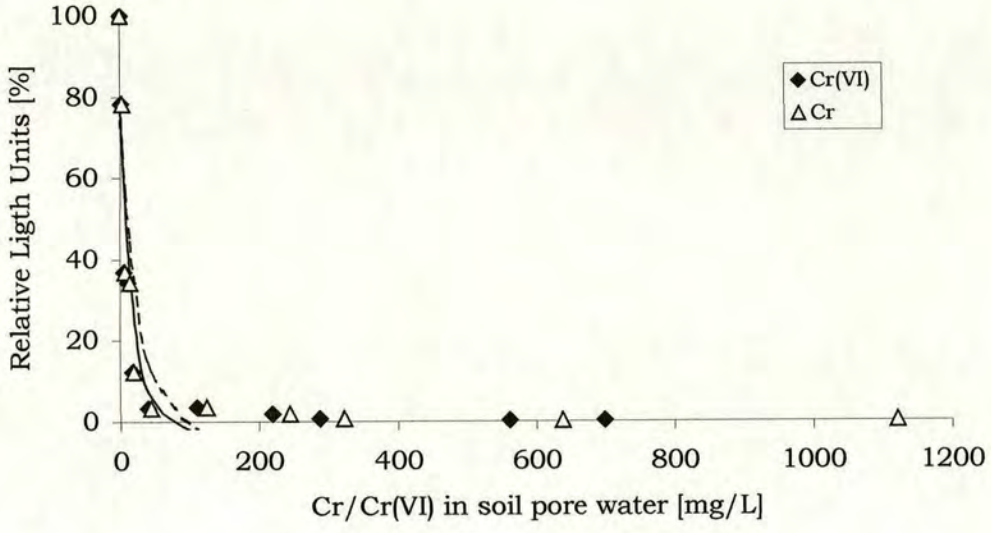


Fig. 5.22 The effect of Cr and Cr(VI) on the luminescence of *E. coli* pUCD607 in soil pore waters obtained from Cr-contaminated Glencorse soils. The control for the assay was the soil pore water with no Cr (*i.e.* microcosm control).

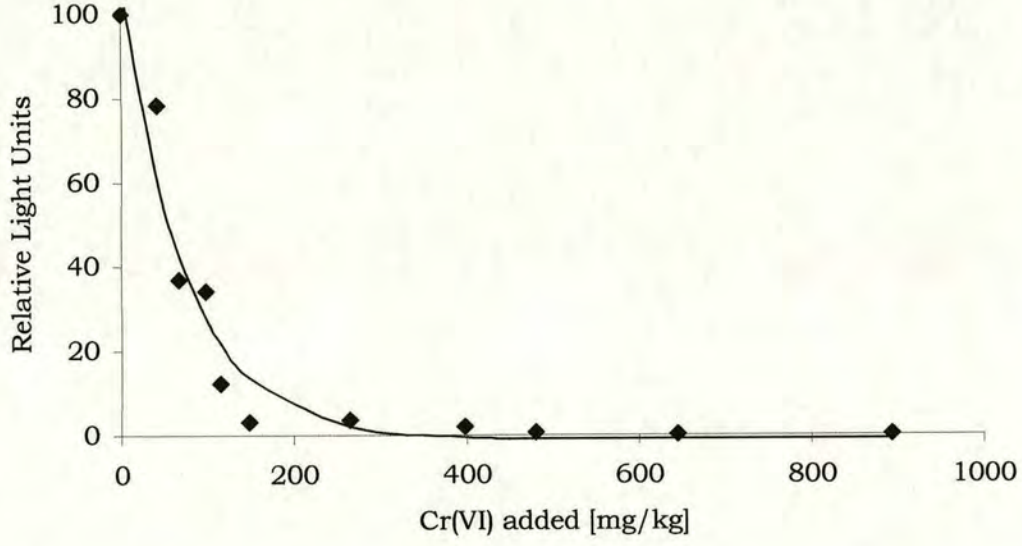


Fig. 5.23 The effect of Cr(VI) added to soils on the *E. coli* pUCD607 luminescence in soil pore waters obtained from Cr-contaminated Glencorse soils. The control for the assay was the soil pore water with no Cr (*i.e.* microcosm control).

EC values (Table 5.8) for all curves in Glencorse were obtained by using the parameters from regression analysis.

Table 5.8 EC values for the toxicity of Cr(VI) to *E. coli* pUCD607 – Glencorse.

Control	Compared to Cr	Cr species	EC_{50}*	EC_{25}*
Experiment	In soil pore water	Cr(VI)	8	1
Reference pH 4.9	In soil pore water	Cr(VI)	6	2
Experiment	In soil pore water	Tot Cr	11	1
Reference pH 4.9	In soil pore water	Tot Cr	9	3
Experiment	Added	Cr(VI)	55	26
Reference 4.9	Added	Cr(VI)	54	24

*Units for EC values compared with Cr added are in mg/kg of soil and mg/L Cr in soil pore water.

5.7.1.4 Comparison of Cr toxicity to E. coli pUCD607 in the three different soils

A regression analysis was carried on the bioassays results, using %RLU as response variate and Cr(VI) added to soils as an explanatory variate. The fitted models (Figure 5.24) and the accumulated analysis of variance obtained from the regression showed:

- a highly significant effect of Cr on the bacterial response ($P<0.001$);
- a highly significant difference in bacterial response between soils ($P<0.001$);
- a significant combined effect of soil and Cr in the bacterial response ($P=0.007$).

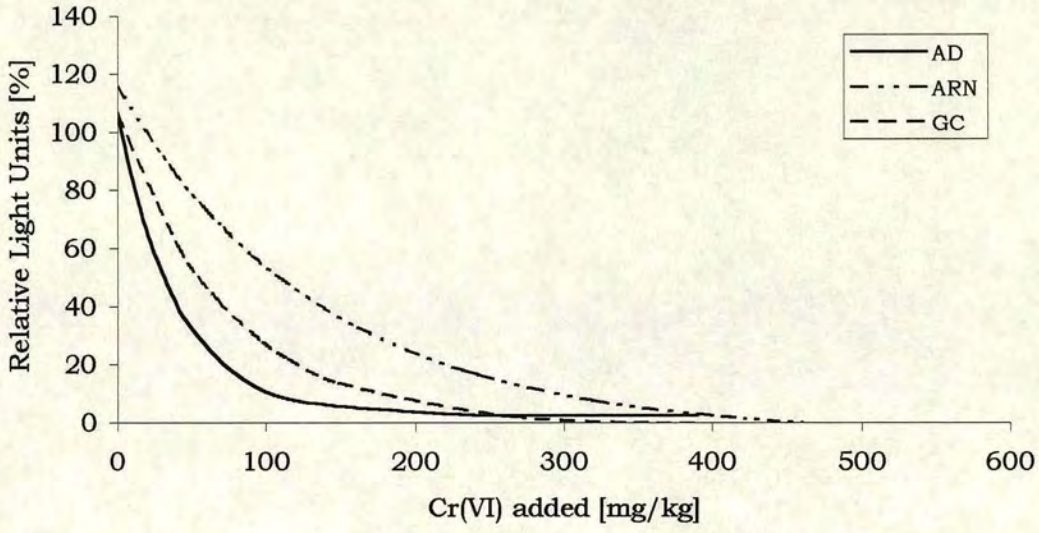


Fig. 5.24 Exponential models obtained from the regression analysis of luminescence response to Cr(VI) added to soils.

From Figure 5.24 and the analysis of EC values obtained for each soil (Table 5.6 to 5.8), it can be concluded that the toxicity of soils to bacteria was in the order Aldroughty > Glencorse > Arnhall, with EC_{50} values of around 30, 50 and 100 mg Cr(VI) added per kg of soil, respectively.

The concentrations of Cr(VI) present in soil solution 30 days after Cr addition to soils are shown in Figure 5.6. It was evident that the concentrations of Cr(VI) in soil solution decreased in the order Aldroughty > Glencorse > Arnhall (with the Cr(VI) adsorption capacities of the same soils increasing in the opposite direction). The results from the bioassays with *E. coli* pUCD607 suggested that, at least for this organism, Cr(VI) was more toxic in soils with the lowest adsorption capacity, supporting the hypothesis formulated in this chapter.

5.7.2 The effect of Cr on soil microbial community structure according to PLFA analysis

Phospholipid fatty acids (PLFAs) were used as indicators of the changes in microbial community structure as a result of Cr contamination. The extraction and analysis of PLFAs was carried out as described in Section 2.3.3.

In total, 37 PLFAs with a chain length up to 20 carbons were detected by GC-MS (Table 5.9). The individual fatty acids were designated in terms of the total number of carbon atoms and the number of double bonds, followed by the position of the double bond from the methyl end of the molecule. *Cis-* and *trans-* configurations are indicated by c and t, respectively. The suffixes *a* and *i* indicate *anteiso* and *iso* branching, respectively; *br* indicates unknown branching and *cy* indicates a cyclopropane fatty acid. The presence of a methyl group on the 10th carbon atom from the carboxyl end of the molecule is indicated by 10Me (Frostegård *et al.*, 1994). A comprehensive compilation of PLFAs that form the guilds plant roots, algae, protozoa, fungi, vesicular arbuscular mykorriza, cyanobacteria and bacteria are included in Laczko *et al.* (1997).

Table 5.9 PLFAs identified in the studied soils.

12:0	16:1i	17:0i	18:2ω6,9	19:0cy
13:0	16:0i	17:0ai	18:1ω9	19:0
14:0i	16:1ω9	17:1ω8	18:1ω7	20:4ω6
14:0	16:1ω7	17:0y	18:1	20:5
15:0i	16:1ω5	18:0br	18:0	20:0
15:0ai	16:0	17:0(10Me)	19:1a	
15:0	17:0br	18:3(5,10,12)	18:0(10Me)	
16:0br	16:0(10Me)	18:2(4/5,10)	19:1br	

5.7.2.1 Community structure of the non-contaminated soils (controls)

The untreated soils (controls) contained a variety of saturated, unsaturated, methyl branched and cyclopropane PLFAs (Figure 5.25). The PLFAs identified in the controls varied significantly between soils (P=0.01). The proportion (as expressed by mol percentage of the total PLFAs for each control) of some PLFAs was higher for Aldroughty than for the other two soils, including saturated PLFAs 15:0, 16:0, 18:0 and 20:0; the unsaturated 18:2ω6,9, 18:1ω9, 18:3(5,10,12), 18:1 and 19:1a. This group might indicate neutrophile microorganisms. Pennanen *et al.* (1998) found that the relative proportion of 19:1a was larger in soils with more neutral pH than in acidic ones, but they also found that 16:1ω5c behaved similarly. In this experiment, 16:1ω5c was found in the same relative amounts in the three soils. PLFA 14:0 was found in Arnhall and Glencorse but was not detected in Aldroughty. The PLFA 18:2(4/5,10) was not found in any of the soils.

It is commonly accepted that 18:2ω6,9 is a reliable indicator of fungal biomass (Frostegård and Baåth, 1996; Fritze *et al.*, 2000). Aldroughty had more relative fungal PLFA than the other two soils. Other authors have reported higher relative concentrations of this PLFA in soils with more acidic pH values and higher organic matter than in neutral ones [Frostegård *et al.*, 1993).

The PLFAs 16:0, 18:1ω9, 18:1ω7 and 19:0cy were found in the highest concentrations in the three soils.

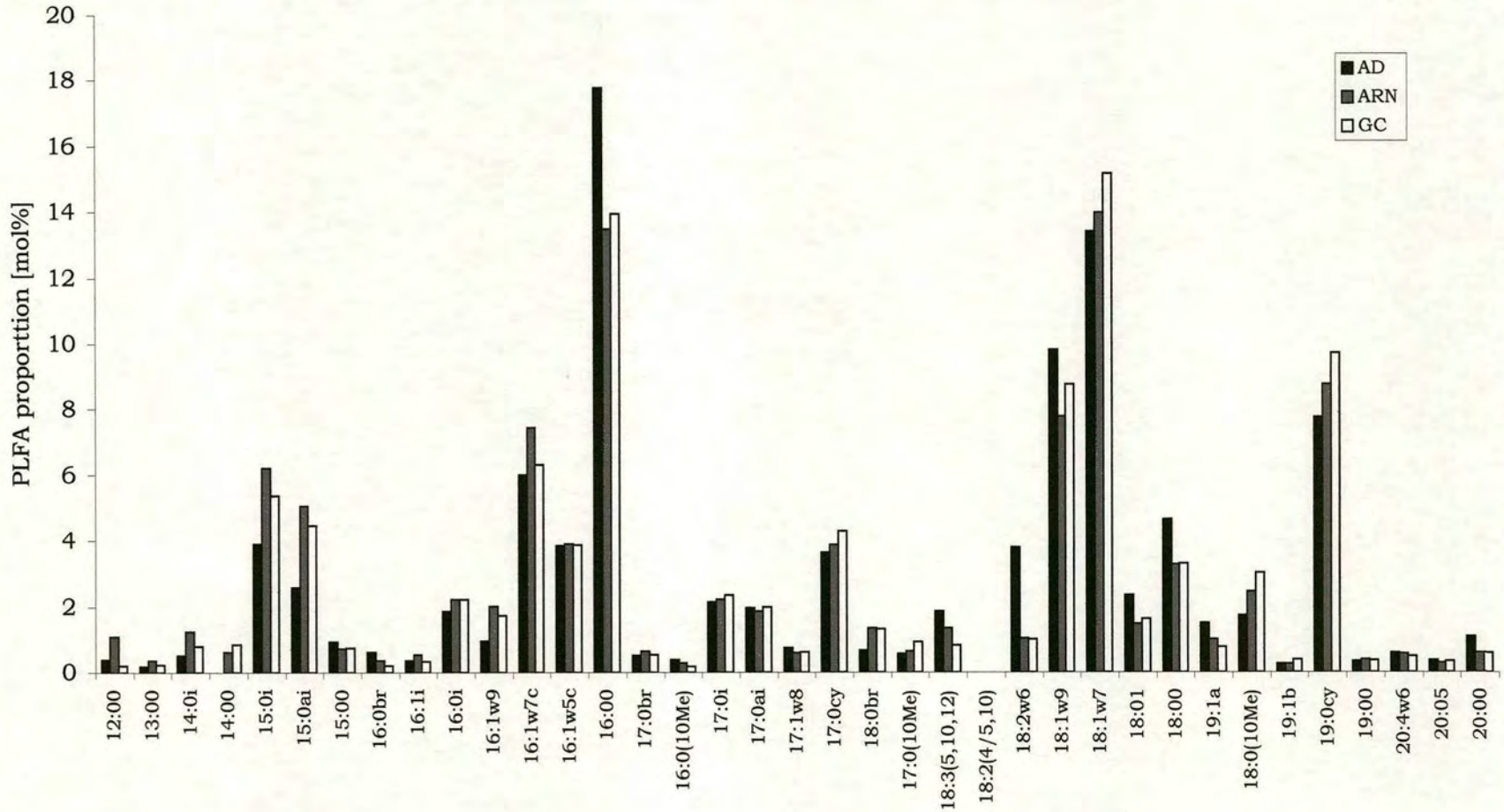


Fig 5.25 PLFA composition (percentage) of the three controls (non-contaminated soils). Differences in the community structure were observed.

5.7.2.2 PLFA patterns and the effect of Cr

In order to compare a large set of samples it is useful to condense the information provided by the individual PLFA patterns. This can be achieved by using multivariate analysis. Principal component analysis (PCA) has been successfully used in PLFA studies (Fritze *et al.*, 2000; Ibekwe and Kennedy, 1998) to identify treatment effects on the soil microbial community structure.

In this case the PCA analysis on the relative PLFA (mol%) was used to identify any possible groupings and patterns in the microbial communities related to the addition of Cr(VI) to the soil. The grouping of the samples was visualised with scatter diagrams of the scores of the second component (PC2) *versus* the first component (PC1). The analysis of the latent roots from the covariance matrix indicated that 85.94 % of variation was explained by the first four components, with the first two components contributing the most (72%).

The plot of the scores of PC2 *versus* the scores of PC1, using soil as a treatment (Figure 5.26), showed distinctive groupings for each soil, indicating differences in the microbial community. The difference between Aldroughty and the two other soils is clearly shown. The plot of PC4 *versus* PC3 did not further resolve any differences between the groupings (plot not shown).

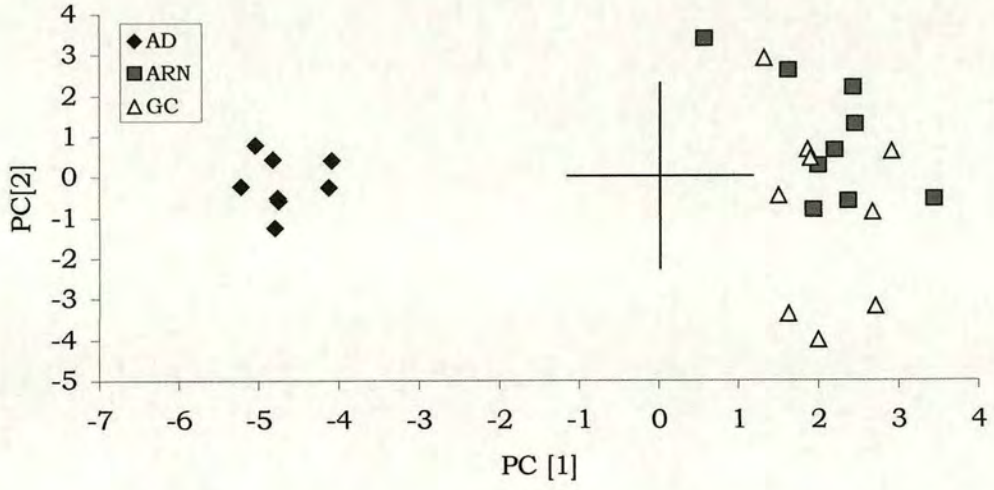


Fig. 5.26 Plot of the scores of PC 2 *versus* the scores of PC 1 using soil as a treatment. The separation between Aldroughty and the two other soils is clear. The PCA was carried out on PLFA mol%.

The scores of the PC1 obtained from the PCA using data in nmol/g were plotted *versus* Cr(VI) added, Cr(VI) in soil pore waters and soil pH (Figure 5.27). Figure 5.27a and 5.27b show that Arnhall and Glencorse PC1 scores decreased exponentially with the increase of Cr(VI) added and Cr(VI) in soil pore water, respectively, but that there was no change for Aldroughty. Figure 5.27c shows linear decrease of PC1 scores from Arnhall and an almost linear decrease of scores from Glencorse with the increase of soil pH. The scores of Aldroughty did not change with pH.

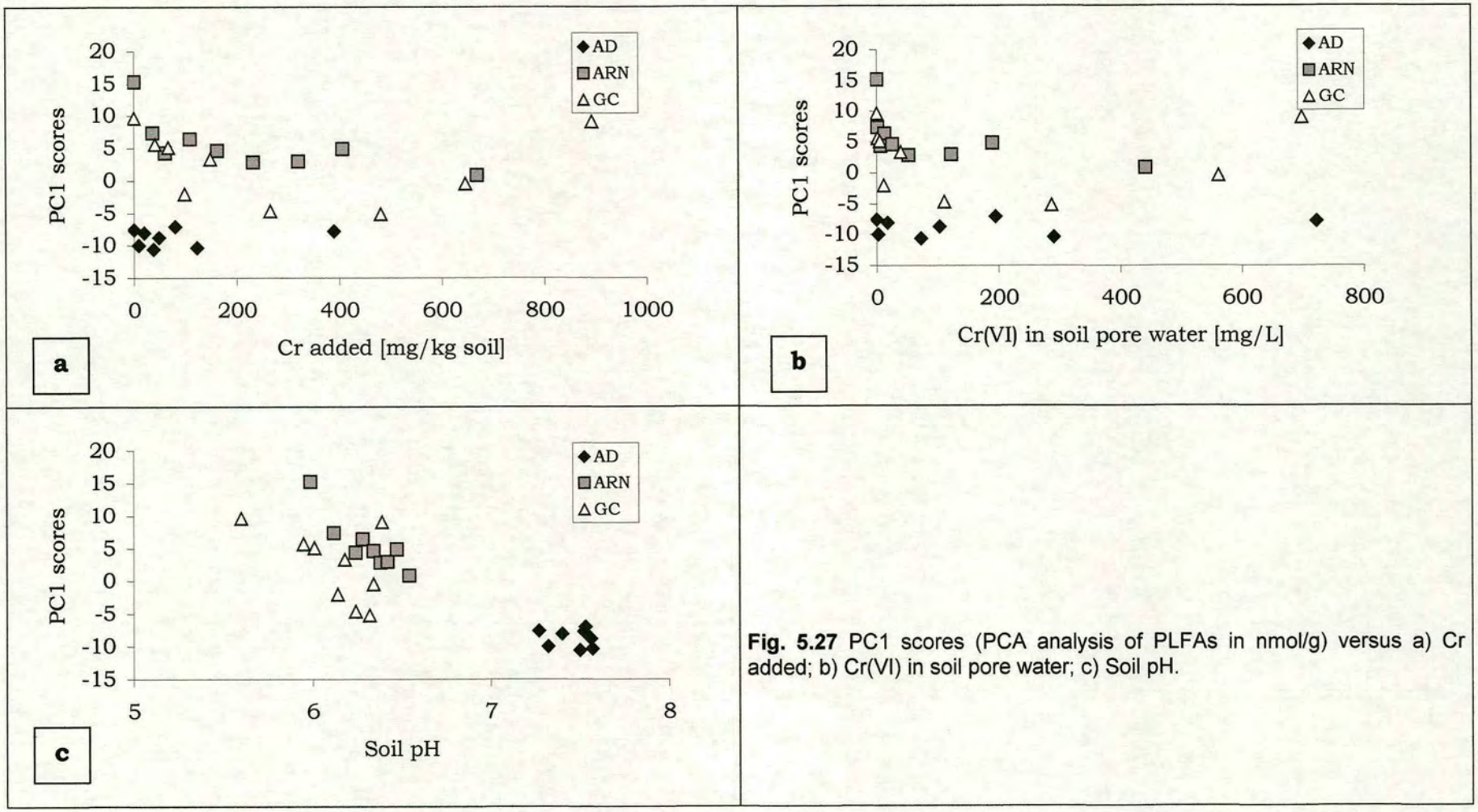


Fig. 5.27 PC1 scores (PCA analysis of PLFAs in nmol/g) versus a) Cr added; b) Cr(VI) in soil pore water; c) Soil pH.

The regression analysis on PC1, using Cr added, Cr(VI) in soil pore water and soil pH as treatments, showed a significant to highly significant effect of the three variables in the PC1 scores – Cr(VI) added ($P<0.001$), Cr(VI) in soil solution ($P=0.037$), soil pH ($P<0.001$). There were also highly significant differences in the PC1 scores between soils and the three variables (all $P<0.001$). The Cr(VI) added, Cr(VI) in soil pore waters and soil pH explained the variation in the regression in a similar manner – Cr(VI) added, 96.8%; Cr(VI) in soil solution, 96.9%; and soil pH, 96.7%.

The PCA clearly shows clearly a separation of soils in three distinctive groups, with Arnhall and Glencorse being similar but still distinguishable. It also showed that Cr added, Cr(VI) in soil pore waters and soil pH had an effect on the scores, between and within soils. These three factors seem to have an effect on the PLFAs found in soils and therefore on the toxicity of Cr-contaminated soils.

The total amount of microbial PLFAs in each sample was used as an indicator of the total microbial biomass (Pennanen *et al.*, 1998). This was calculated from the sum of the concentrations of the 37 individual PLFAs. When comparing the three soils (Figure 5.28), the difference in total PLFAs was highly significant ($P<0.001$). The difference in microbial biomass between Aldroughty and the two other soils was very obvious, and as expected (due to the low content of organic matter), Aldroughty had a low biomass. In the unamended control soils, Aldroughty had a much lower total PLFA than the other two soils. The difference in total PLFAs between Arnhall and Glencorse was slightly significant ($P=0.1$).

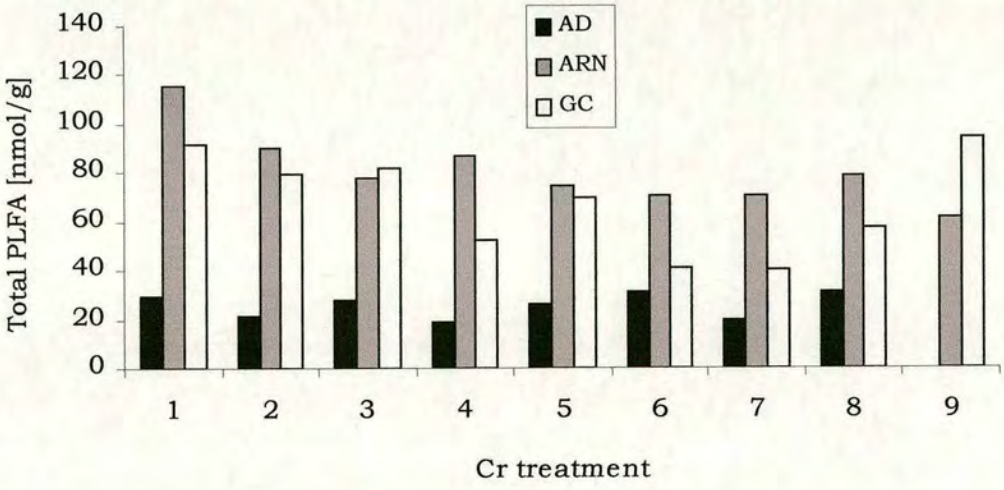


Fig. 5.28 Comparison of microbial biomass (total PLFAs) in Aldroughty, Arnhall and Glencorse after 30 days incubation with increasing concentrations of Cr(VI). Treatment 1 had no Cr and treatment 9 had the highest amount of Cr added.

In Figure 5.29 and 5.30 the total amount of PLFAs was plotted *versus* Cr(VI) added and Cr(VI) in soil pore waters, respectively. The effects of Cr(VI) added to soils and Cr(VI) in soil pore waters on total PLFAs were similar. The increase of Cr(VI) added and higher Cr(VI) in soil pore waters resulted in a decrease of total PLFA in Arnhall and Glencorse, but the change was not very obvious for Aldroughty.

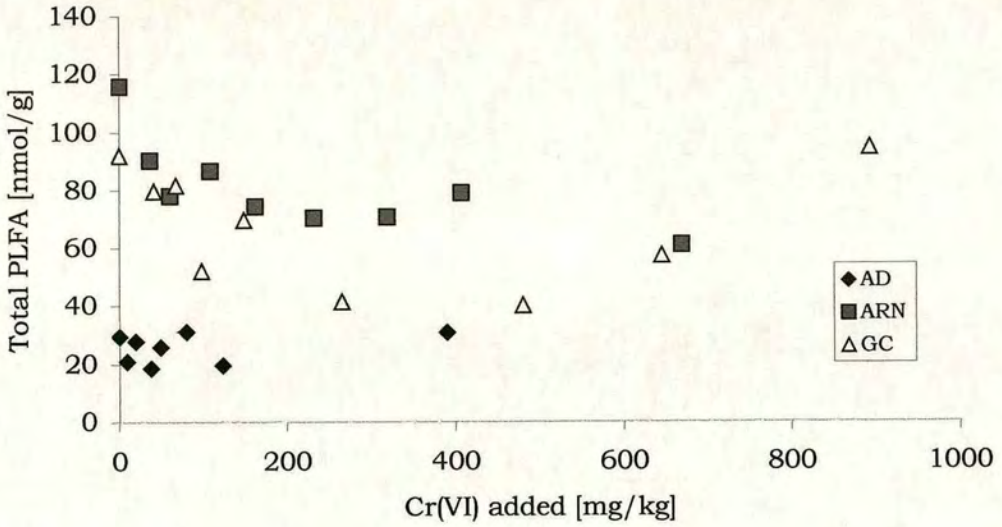


Fig 5.29 The effect of Cr(VI) added to soil on the total amount of PLFAs in Aldroughty, Arnhall and Glencorse.

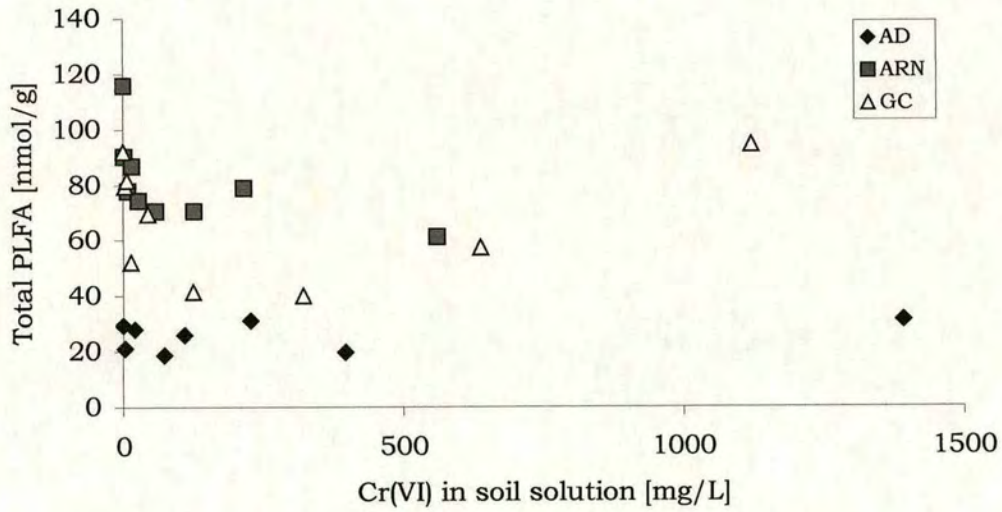


Fig. 5.30 The effect of Cr(VI) in soil pore waters on the total amount of PLFAs in Aldroughty, Arnhall and Glencorse.

The bacterial PLFAs were calculated from the sum of characteristic PLFAs for bacteria (Yao *et al.*, 2000), 15:0i, 15:0a, 15:0, 16:0i, 16:0, 17:0i, 17:0a, 17:0cy, 18:0, and 19:0cy (Frostegård and Bååth, 1996). Frostegård *et al.* (1993) suggested that there is still some disagreement as to which PLFAs should be considered exclusively of bacterial origin. Bacterial PLFAs accounted for 40 to 50% of the PLFAs measured. The changes in bacterial PLFAs with the addition of Cr(VI) to soil is plotted in Figure 5.31. Significant negative relationships were clearly observed for Arnhall and Glencorse. Above 500 mg/kg of Cr(VI) added, an increase in bacterial PLFAs was observed, probably because soils were approaching more neutral pH.

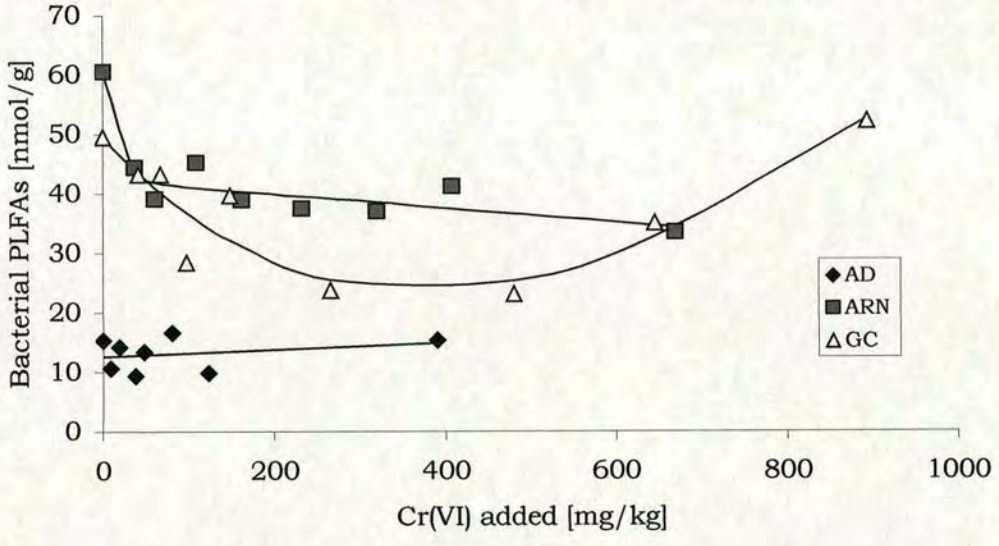


Fig. 5.31 The effect of Cr(VI) added to soil on the bacterial PLFAs of Aldroughty, Arnhall and Glencorse.

The same patterns are observed when comparing the change of bacterial PLFAs and the content of Cr(VI) in soil pore waters (Figure 5.32).

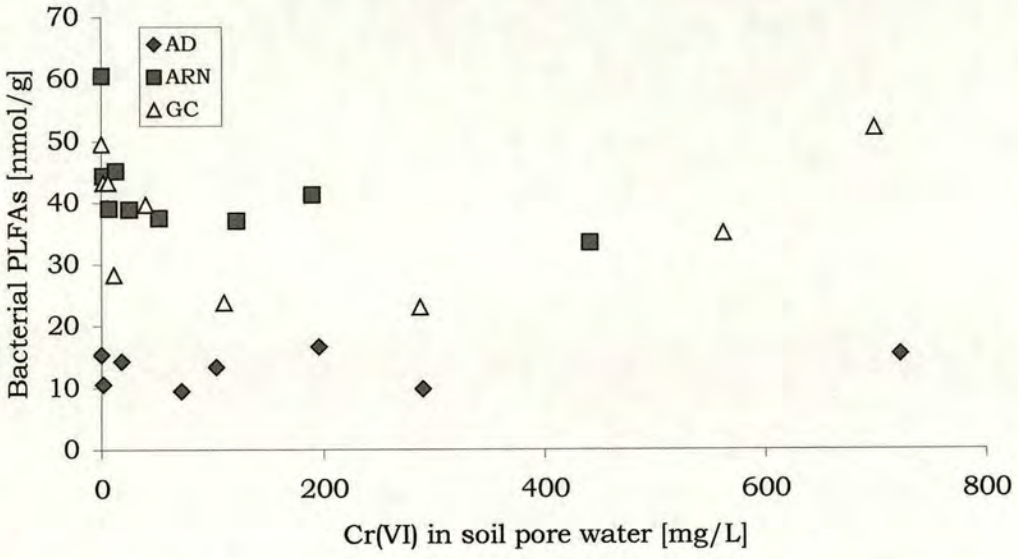


Fig. 5.32 The effect of Cr(VI) in soil pore waters on the bacterial PLFAs of Aldroughty, Arnhall and Glencorse.

Phospholipid fatty acids predominantly present in Gram-negative bacteria (16:1ω5, 16:1ω7, 16:ω9, 17:0cy, 18:1ω5, 18:1ω7 and 19:0 cy) were found in higher amounts in these soils than PLFAs of Gram-positive origin (16:0(10 Me), 17:0(10Me), 18:0(10Me), 15:0i, 15:0a, 16:0i, 17:0i and 17:0a), with Aldroughty having the highest amount of Gram-negative bacteria. The ratio of Gram-positive to Gram-negative bacteria was calculated by taking the sum of the predominant Gram-positive PLFAs, and dividing by the sum of predominant Gram-negative bacterial PLFAs (Yao *et al.*, 2000). The change in this ratio was not very marked with the addition of Cr(VI) in Aldroughty and tended to increase with Cr(VI) concentration in Arnhall and Glencorse (Figure 5.33).

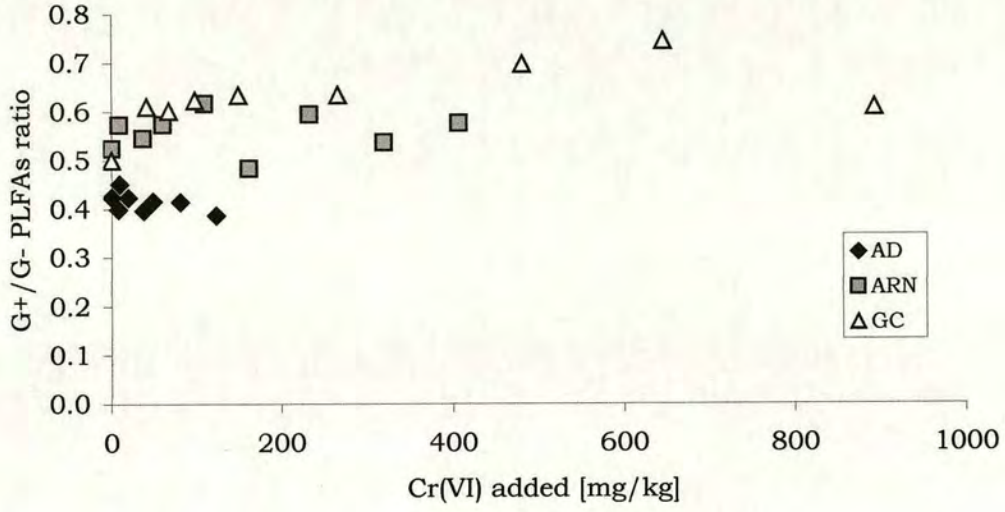


Fig. 5.33 The effect of Cr(VI) added on the Gram-positive to Gram-negative bacterial PLFAs ratio (G+/G-) of Aldroughty (AD), Arnhall (ARN) and Glencorse(GC).

The effect of Cr(VI) added on the PLFAs from Gram-positive bacteria was very similar to that of the bacterial PLFA (Figure 5.34). The same trend was observed in the PLFAs from Gram-negative bacteria (Figure 5.35).

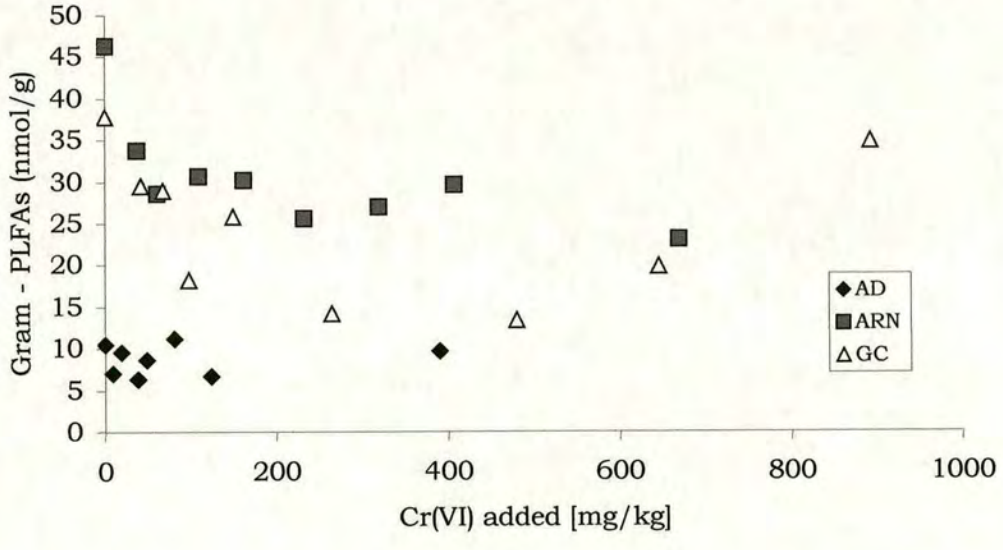


Fig. 5.34 The effect of Cr(VI) added to soil on the Gram-negative bacterial PLFAs of Aldroughty, Arnhall and Glencorse.

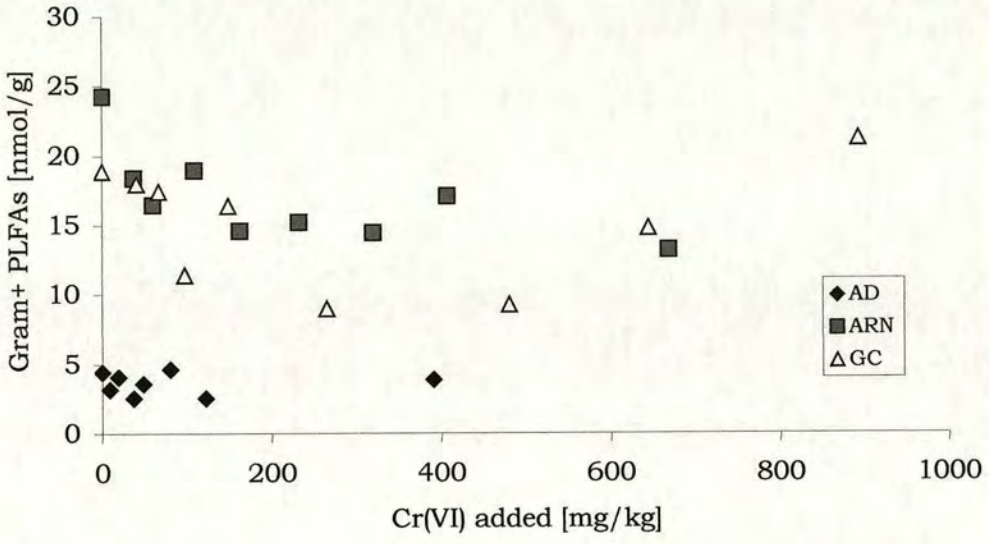


Fig. 5.35 The effect of Cr(VI) added to soil on the Gram-positive bacterial PLFAs of Aldroughty, Arnhall and Glencorse.

The PLFAs 16:0(10 Me), 17:0(10Me), and 18:0(10Me) were considered as representative of the actinomycetes. A very low relative abundance of these PLFAs was found compared to the bacterial PLFAs. When Cr(VI) was added to soils, the actinomycetes community behaved in a similar way to bacteria (Figure 5.36). There was no evident change in Aldroughty, but in Arnhall and Glencorse, these PLFAs decreased with the increase in Cr. In the Glencorse soil, an increase in these PLFAs was observed when pH values approached neutrality.

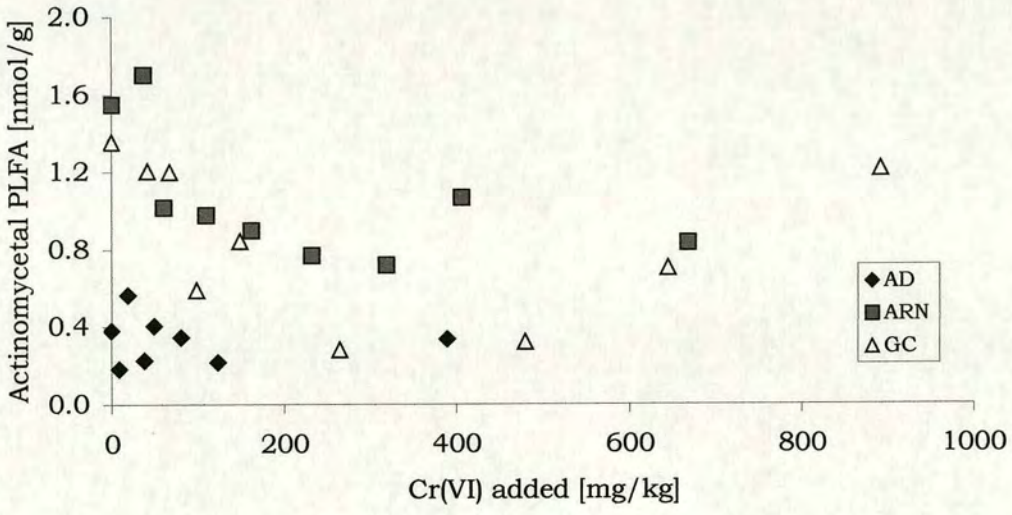


Fig 5.36 The effect of Cr(VI) added to soil on the actinomycetal PLFAs of Aldroughty, Arnhall and Glencorse.

Fungal PLFAs (18:2ω6,9) (Frostegärd and Bääth, 1996) showed a completely different pattern to other PLFAs (Figure 5.37). In Aldroughty they decreased with the addition of Cr(VI). In Arnhall they increase with the additions of Cr(VI) up to 110 mg/kg and decreased again. In Glencorse the fungal PLFA increased with the addition of Cr(VI) up 70 mg/kg, decreased with Cr(VI) up to 480 mg/kg and increased again, probably suggesting shifts towards more resistant fungal species.

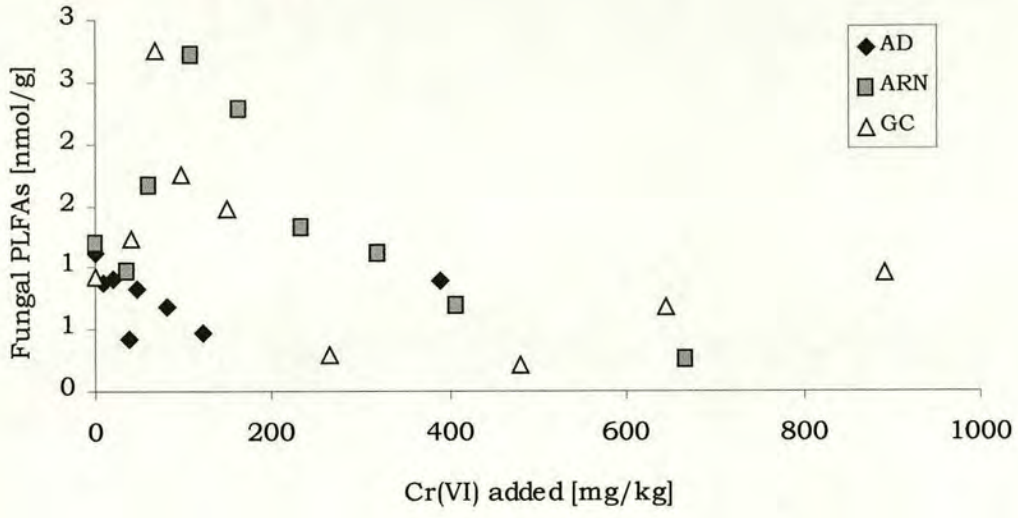


Fig. 5.37 The effect of Cr(VI) added on fungal PLFAs of Aldroughty (AD), Arnhall (ARN) and Glencorse(GC).

In order to calculate EC values for the toxicity of Cr(VI) added to the different microbial groups discussed previously, concentration-response curves were prepared by using percentage PLFA related to the relevant soil control. The percentage of PLFA for individual treatments and groups of PLFAs were calculated, using the non contaminated soil as a reference (i.e. the control = 100% of PLFA for each guild). Concentration-response curves were obtained for Arhall and Glencorse, but not for Aldroughty, the changes in PLFAs did not follow a particular trend. Effect concentration values (EC) obtained from the regression curves are included in Table 5.10.

Table 5.10 EC values obtained from plots of PLFA percentage reduction versus Cr(VI) added to soils.

Soil	PLFA guild	EC_{50} Cr(VI) mg/kg*	EC_{25} Cr(VI) mg/kg
Arnhall	Total	NA	47
Glencorse	Total	278	88
Arnhall	Bacterial	NA	31
Glencorse	Bacterial	330	93
Arnhall	Actinomycetes	230	76
Glencorse	Actinomycetes	150	65
Arnhall	Gram+	NA	46
Glencorse	Gram+	378	116
Arnhall	Gram-	NA	29
Glencorse	Gram-	174	56
Arnhall	Fungi	443	348
Glencorse	Fungi	263	213
Arnhall	Eukaryotes	347	256
Glencorse	Eukaryotes	202	158

*NA= not available. These values could not be obtained because the curves did not reach the 50% effect values.

It was not possible to obtain EC_{50} values for all PLFA guilds in each soil, so the EC_{25} values were used to compare the toxicity of Cr(VI) in the two different soils (Figure 5.38). Prokaryotes seemed to be more sensitive to Cr(VI) added in soils than eukaryotes, with the most sensitive guild being Gram-negative bacteria and the least sensitive, fungi.

Chromium (VI) added to Arnhall was more toxic to bacteria than Cr(VI) added to Glencorse. In contrast, the opposite was observed for actinomycetes, fungi and eukaryotes.

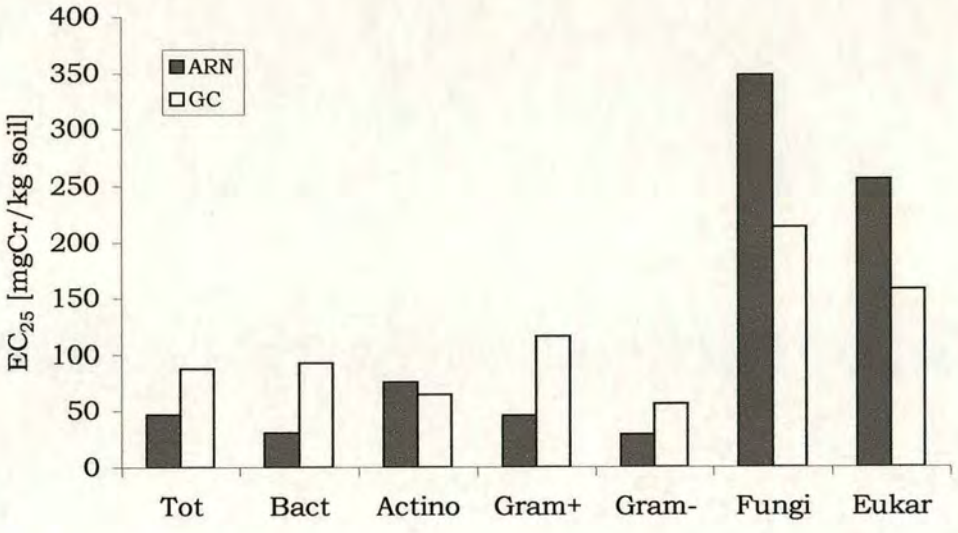


Fig. 5.38 Comparison of EC_{25} values for Cr(VI) toxicity to different microbial groups in Arnhall and Glencorse. Tot = total PLFA, Bact = bacterial, Actino = actinomycetal, Eukar = eukaryotic).

5.7.3 Effects of Cr(VI) on the metabolic capacity of soil microbial communities

The analysis of PLFAs provides information on the structure of the microbial community related to taxonomic groups but does not provide information on the functional consequences of such change. One of the main soil functions that microorganisms perform is the decomposition of organic compounds and litter. Microorganisms are capable of utilising carbon sources of different types depending on their specialisation and this is an important function underpinning different nutrient cycling processes in soil (Pennanen, 2001).

If the metabolic capacities of soil microorganisms are impaired, soil functioning will potentially be reduced. The metabolic capacity of microorganisms in the soils of this experiment was investigated using a Community Level Physiological Profile test (Dye test, Section

2.3.4), where 15 different carbon sources and water were added to subsamples of the microcosm soils, and the production of carbon dioxide for individual carbon sources (substrate induced respiration, SIR) was measured.

Carbon sources used in the test plates were sugars (L-arabinose, fructose, D-galactose and D-glucose), amino acids (L-alanine, aspartic acid, cysteine, L-lysine and serine) and carboxylic acids (citric acid, γ-amino butyric acid, malic acid, N-acetyl glucosamine, oxalic acid and 3,4-dihydroxybenzoic acid). A blank control with water only added was also included. Each sample was tested in triplicate.

The basal respiration (production of carbon dioxide without the addition of carbon source) and the SIR was measured for each carbon source in the different soils.

The respiration (production of CO_2) of microbial communities in soils without Cr(VI) and carbon source is shown in Figure 5.39. Aldroughty showed the lowest respiration of the three soils.

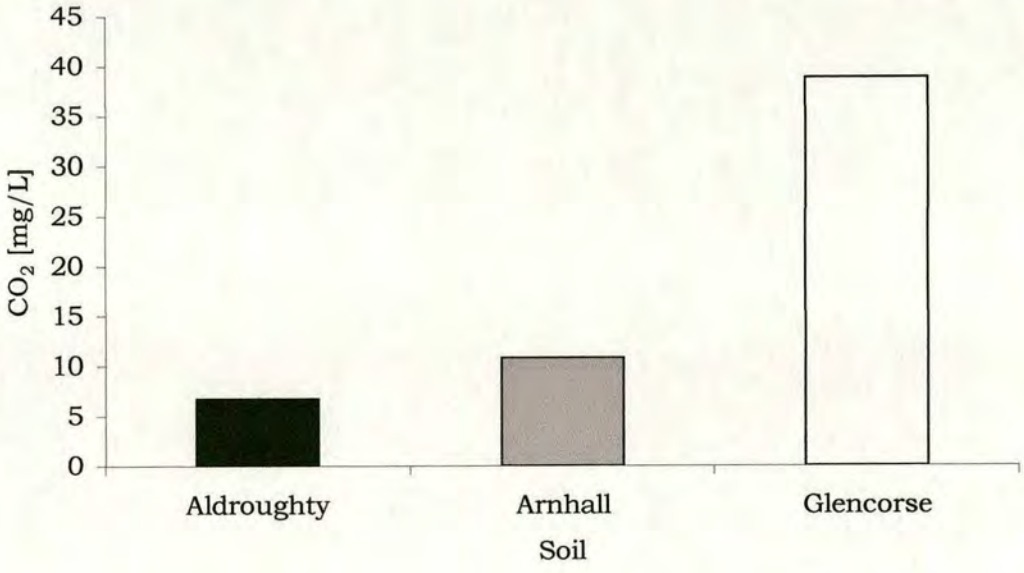


Fig. 5.39 Basal respiration of communities from Aldroughty, Arnhall and Glencorse.

The amounts of carbon dioxide produced by the addition of the different carbon sources were subjected to principal component analysis. In this case the PCA on the relative CO_2 production (CO_2%) was used to identify any possible groupings and patterns in the microbial communities related to the addition of Cr to the soil. The grouping of the samples was visualised with scatter diagrams of the scores of the second component (PC2) *versus* the first component (PC1). The analysis of the latent roots from the covariance matrix indicated that 78.03 % of variation was explained by the first four components.

The plot of the scores of PC2 *versus* the scores of PC1 using soil as a treatment (Figure 5.40), showed distinctive groupings for each soil, indicating differences in the microbial metabolic capacity. The difference between Aldroughty and the two other soils is clearly shown. The plot of PC4 *versus* PC3 did not further resolve any differences between the groupings (plot not shown).

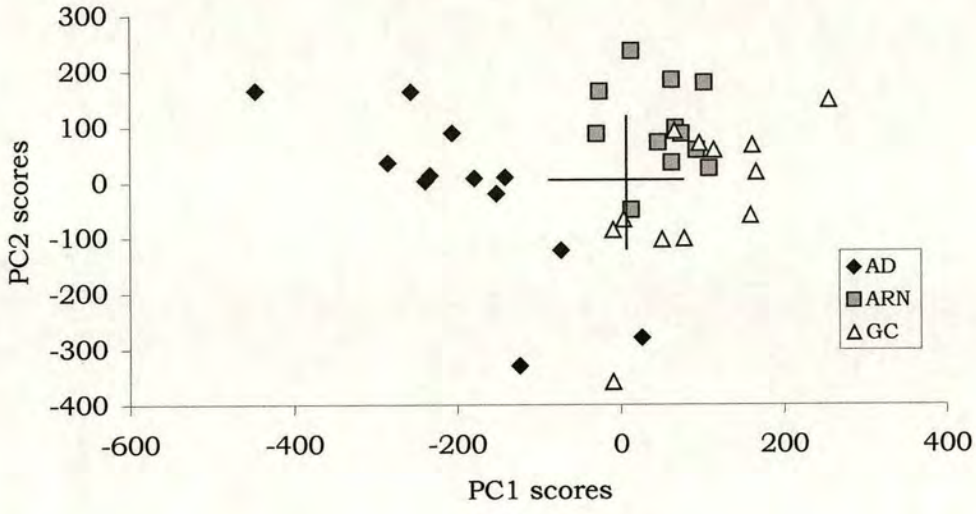


Fig. 5.40 Plot of the scores of PC 2 *versus* the scores of PC 1 using soil as a treatment. The separation between Aldroughty and the two other soils is clear. The PCA was carried out on CO_2 rate%.

The scores of the PC1 were plotted *versus* Cr(VI) added, Cr(VI) in soil pore waters and soil pH (Figure 5.41). Figures 5.41a and 5.41b show that Arnhall PC1 scores decreased with the increase of Cr(VI) added and Cr(VI) in soil pore water, respectively; that there was no change for Aldroughty, and that for Glencorse, the scores increased up to certain Cr(VI) concentrations (115 mg/kg Cr(VI) added to soil and 40 mg/L Cr(VI) in soil pore waters) and then decreased above those concentrations. Figure 5.41c shows the linear decrease of PC1 scores from Arnhall and Glencorse with the increase of pH.

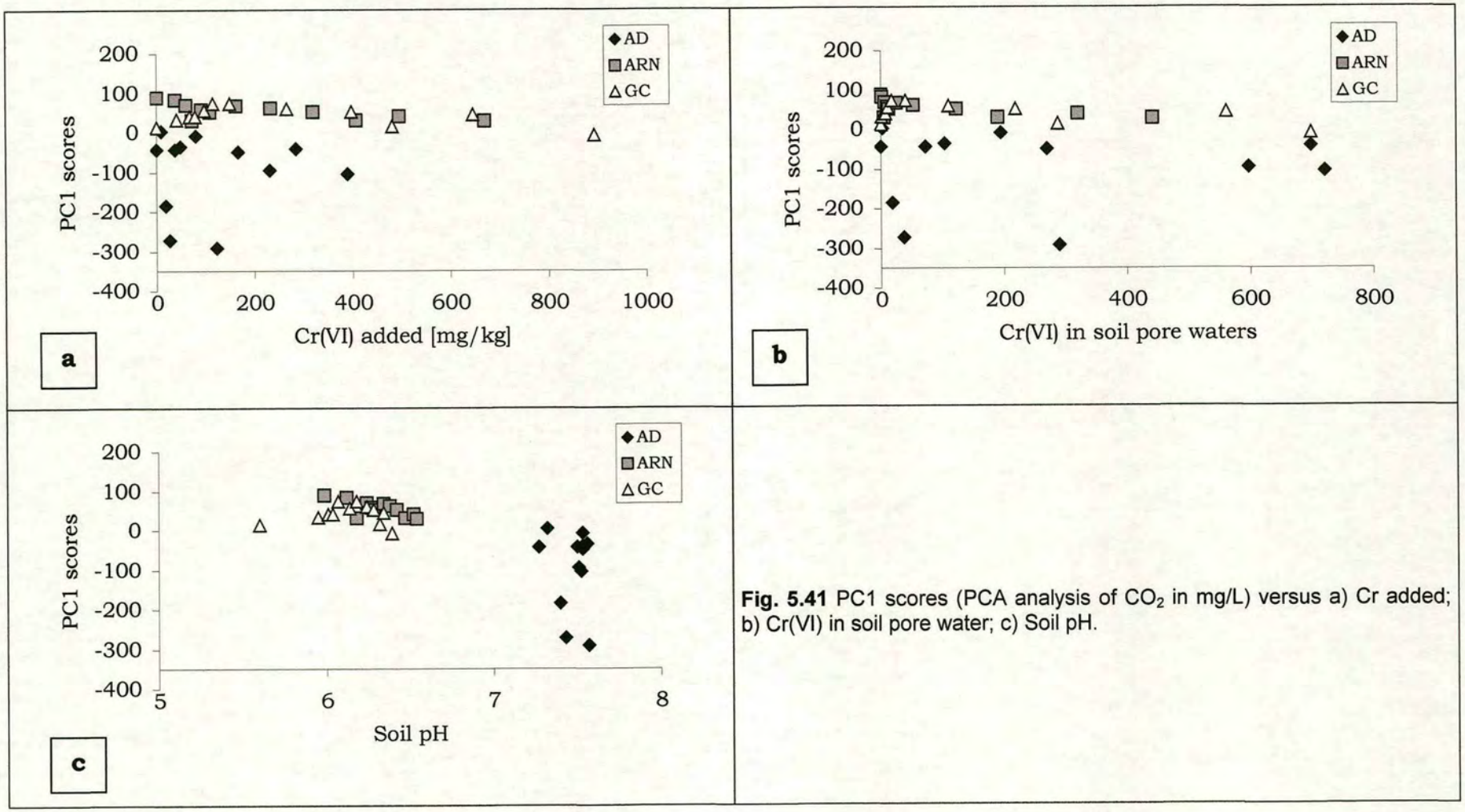


Fig. 5.41 PC1 scores (PCA analysis of CO_2 in mg/L) versus a) Cr added; b) Cr(VI) in soil pore water; c) Soil pH.

The overall production of carbon dioxide was calculated using the sum of individual carbon sources for each treatment as a measure of overall activity. Glencorse showed the highest metabolic capacity in the controls (non-amended soils), followed by Aldroughty. The metabolic capacity of Arnhall in the non-amended soils was very low (Figure 5.42).

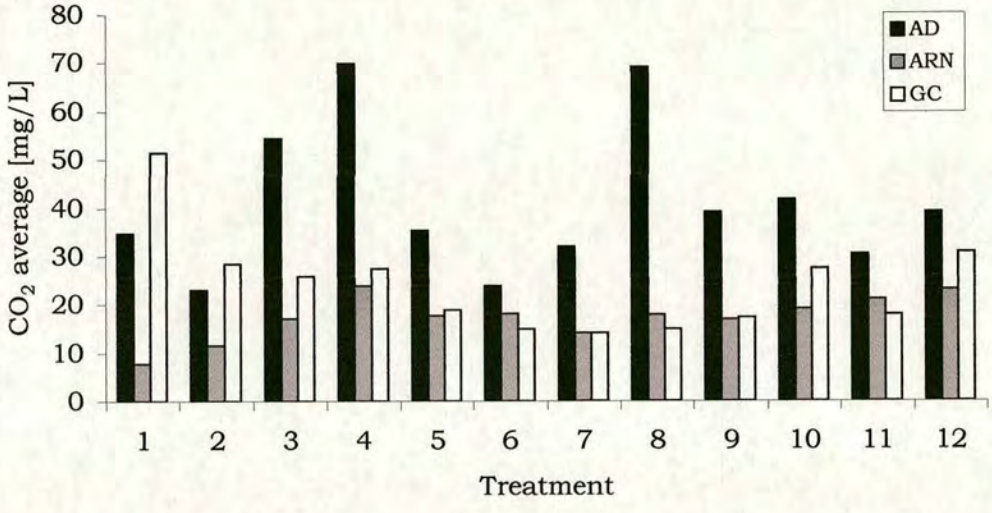


Fig. 5.42 Comparison of production of CO_2 in the three soils and their treatments. Treatment 1 represents the non-amended soil (control) and treatment 12 the highest Cr addition.

The SIR was plotted against Cr(VI) added, Cr(VI) in soil pore water and soil pH (Figure 5.44), from the regression analysis it was found that:

- the effect of Cr(VI) added (Figure 5.43a)on the respiration was highly significant (P=0.004);

- the effect of Cr(VI) in soil pore waters (Figure 5.43b) on respiration was also highly significant (P=0.003);

- the effect of soil pH on respiration (Figure 5.43c) was highly significant ($P<0.001$);

- the difference in the respiration for the three soils was highly significant ($P<0.001$);

- the variable that explained the best variation was soil pH (66.4%), followed by Cr(VI) added (63.9%), and Cr(VI) in soil pore water 51.8%.

The average of the CO_2 production for each carbon source was taken and used to compare the SIR within and between soils types (Figure 5.44). The highest metabolic activity was recorded for Aldroughty and the lowest for Arnhall.

Figure 5.44 shows that the carbon sources which produced the highest amounts of CO_2 in Aldroughty soils were cysteine, N-acetylglucosamine and oxalic acid. For Arnhall and Glencorse the highest respiration was recorded for fructose and the respiration produced by cysteine was low compared with that of Aldroughty.

The regression analysis on respiration in individual carbon sources (data not shown) suggested that, for some carbon sources, soil pH had a highly significant effect in the metabolic capacity of the soils, *e.g.* for cysteine ($P<0.001$), where Cr(VI) added or in soil pore water also had a slightly significant effect ($P=0.099$).

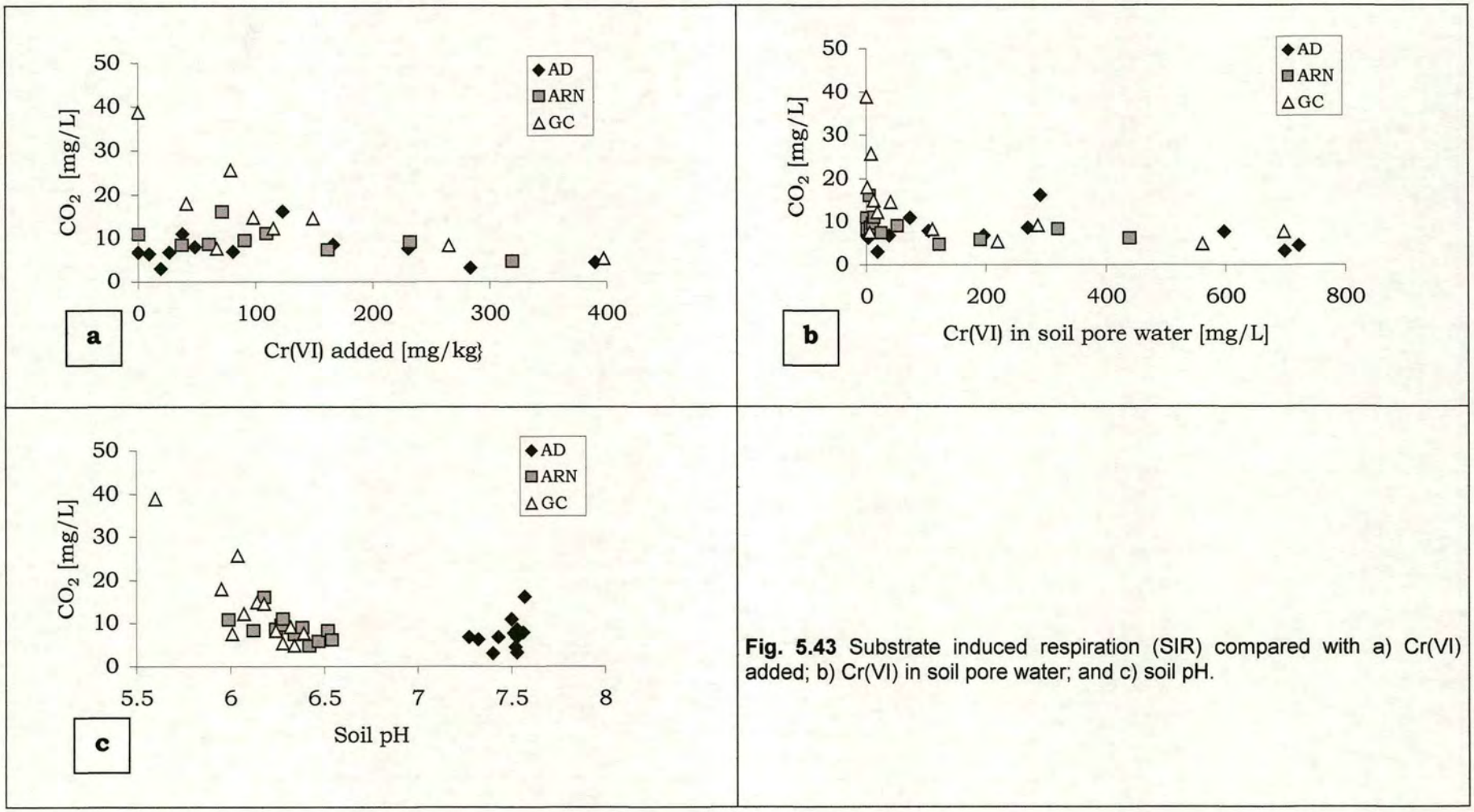


Fig. 5.43 Substrate induced respiration (SIR) compared with a) Cr(VI) added; b) Cr(VI) in soil pore water; and c) soil pH.

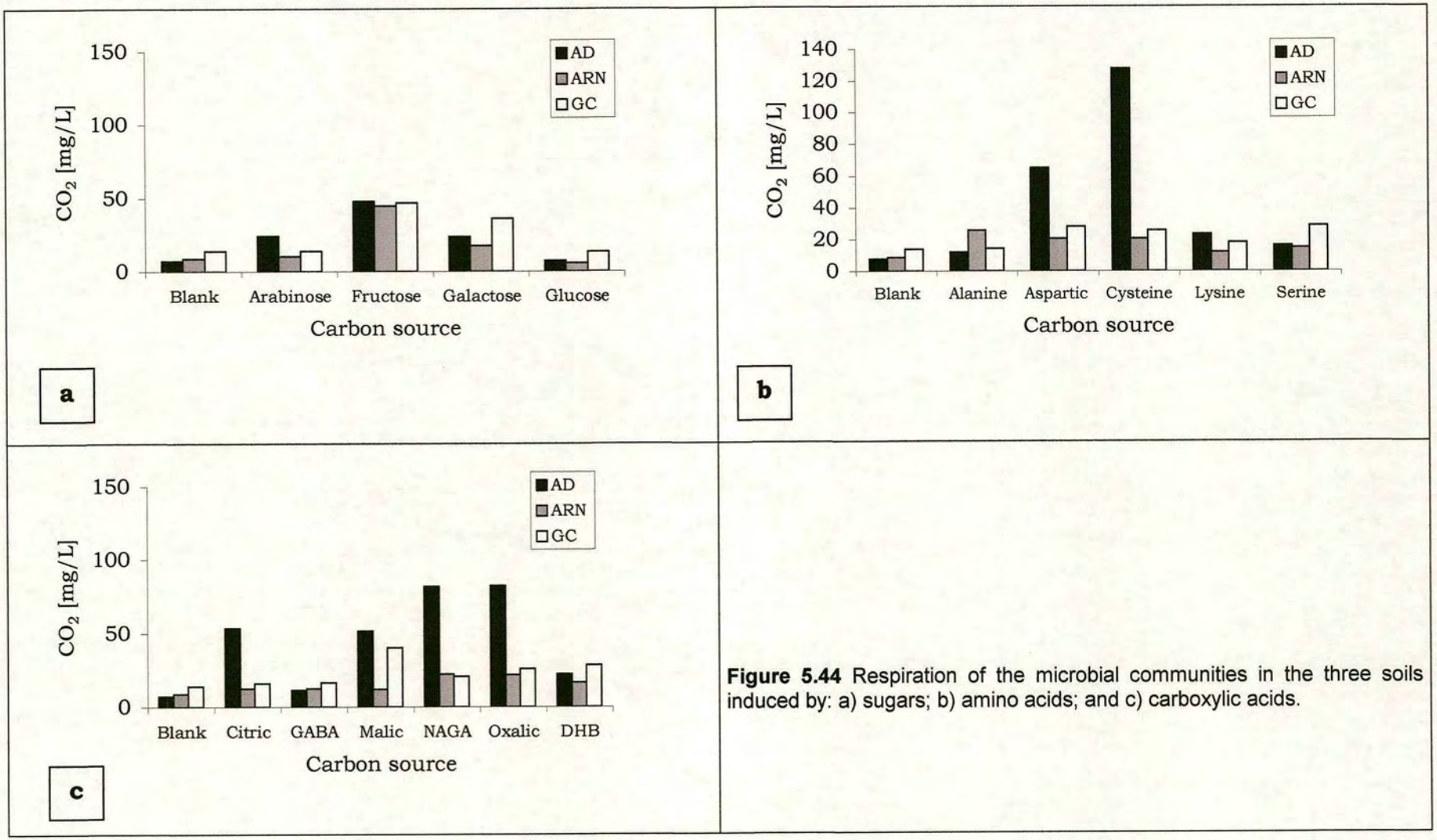


Figure 5.44 Respiration of the microbial communities in the three soils induced by: a) sugars; b) amino acids; and c) carboxylic acids.

5.7.4 Plant assays

In order to compare the effect of Cr at different trophic levels, another single species bioassay based in the inhibition of root growth in barley (*Hordeum vulgare*) was used. The method is described in Section 2.3.5. Due to the small amounts of soil remaining after the soil pore water extraction, the bioassays could only be carried out on Aldroughty and Arnhall.

Seedlings were planted into the soils used for the microcosm experiment (including control soils) and the pots were kept under controlled conditions for five days, after which the root lengths of the seedling were measured and compared with the root lengths in the control soils. The percentage of root length was calculated using the control as 100%. They were plotted against the Cr(VI) added (Figure 5.45) and Cr(VI) in soil pore waters (Figure 5.46) in order to compare with the assays described in previous sections.

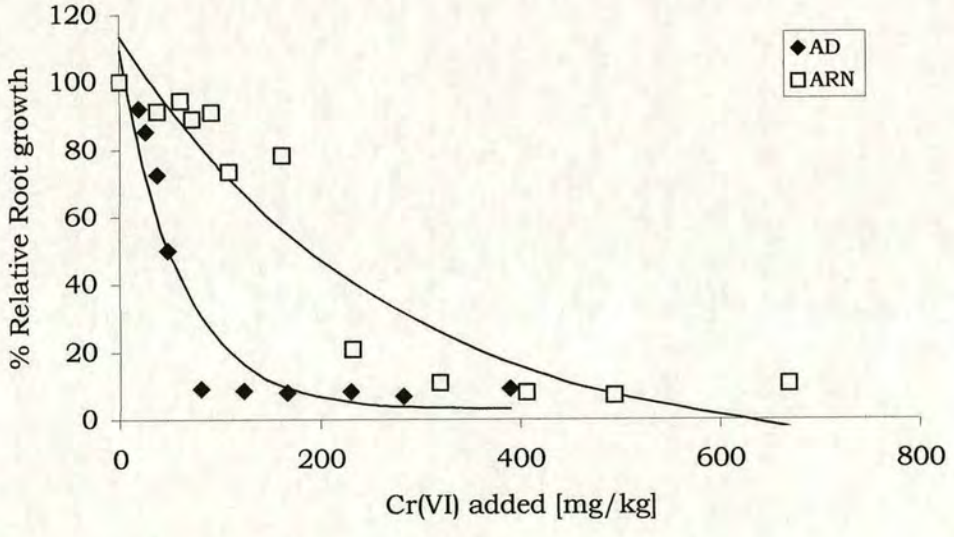


Fig. 5.45 The effect of Cr(VI) added on the relative root growth of *Hordeum vulgare* in two soils.

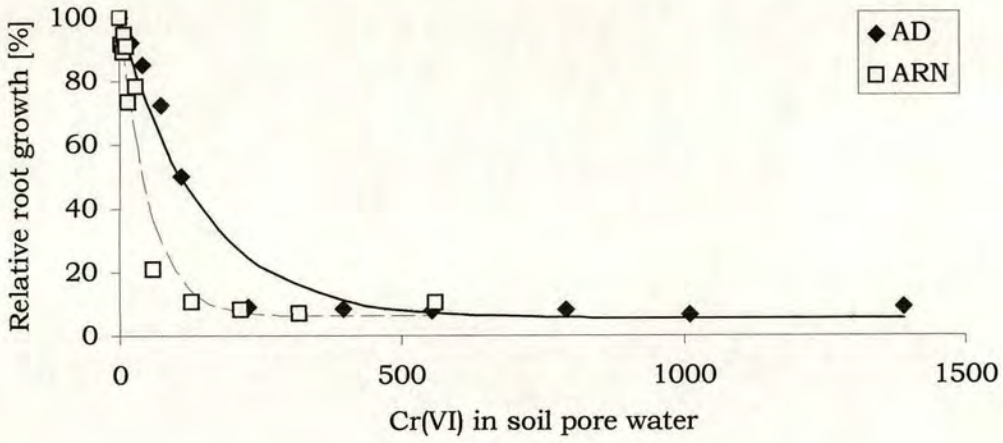


Figure 5.46 The effect of Cr(VI) in soil pore waters on the relative root growth of *Hordeum vulgare* in two soils.

The regression analysis on root growth using Cr(VI) added, explained 89.3 % of the variation, showing a highly significant effect of Cr(VI) added and a highly significant effect of soil type on the percentage root growth ($P<0.001$). The regression analysis on root growth, using Cr(VI) in soil pore waters, explained 95.1 % of the variation, and showed a highly significant effect of Cr(VI) ($P<0.001$) and soil type ($P=0.005$) on the percentage root growth.

The regression analysis on root growth using soil pH (Figure 5.47), explained 68.4% of the variation. The effects of soil pH and soil type were significant ($P=0.011$, $P<0.001$, respectively).

The regression analysis on root growth using soil pore water pH (Figure 5.48), explained 54.2% of the variation. The effect of soil pore water pH and soil type was significant ($P=0.005$, $P<0.001$ respectively).

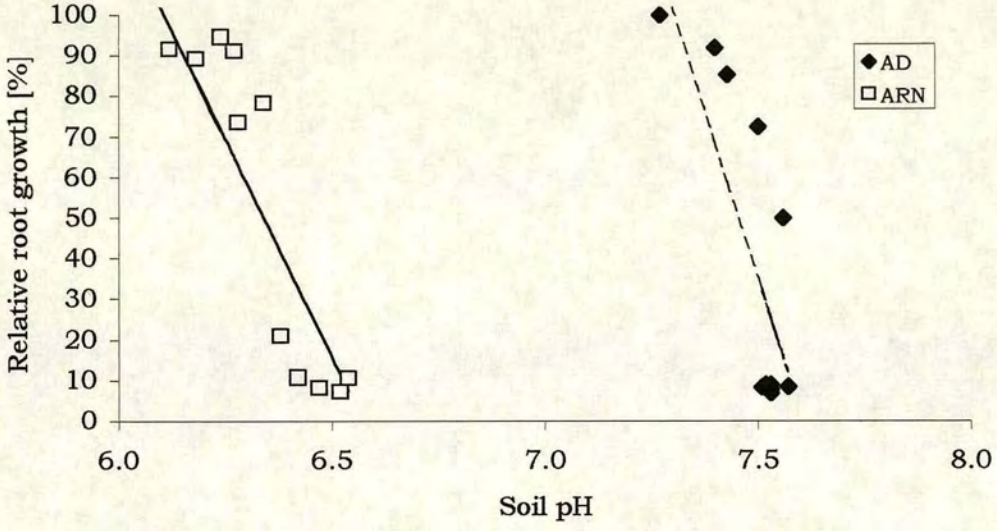


Fig. 5.47 The effect of soil pH on root growth of *Hordeum vulgare* in two soils.

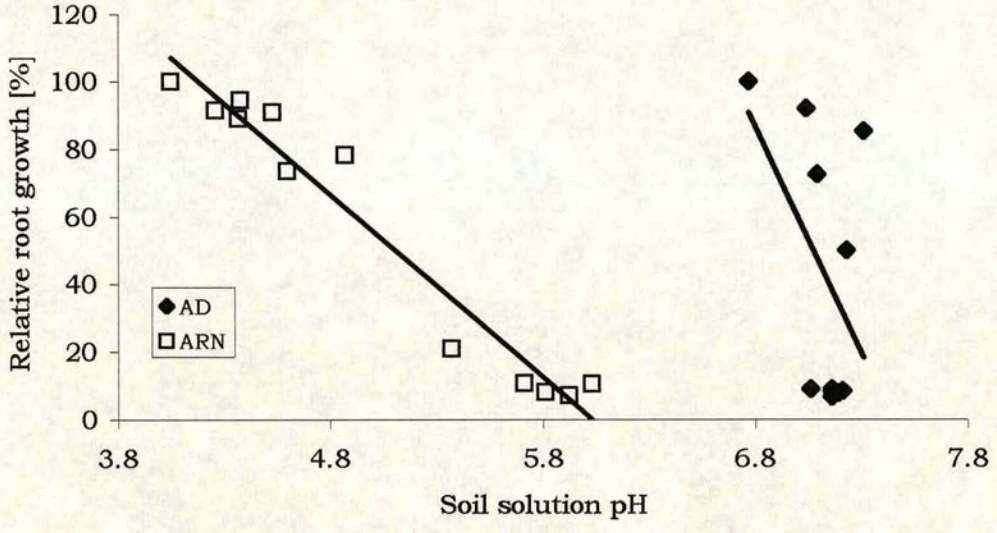


Fig. 5.48 The effect of soil pore water pH on root growth of *Hordeum vulgare* in two soils.

These results showed that the main factors controlling root growth were Cr(VI) added and in soil solution, with pH effects being less important. EC values obtained for these assays are included in Table 5.11. These values might indicate that Cr(VI) will be more toxic for *Hordeum vulgare* when present in a soil with lower Cr(VI) adsorption capacity.

Table 5.11 EC values for Cr(VI) in root length assay for Aldroughty and Arnhall.

Soil	Compared with	EC_{50} (mg/kg)	EC_{25} (mg/kg)
Aldroughty	Cr(VI) added	49	24
Arnhall	Cr(VI) added	188	98

5.8 Discussion

The Cr(VI) adsorption capacity of four soils was investigated and the two surface Langmuir equation was used to calculate the possible concentration of Cr(VI) in soil solution when adding specified amounts of Cr(VI) to soils. Three of those soils were selected to test the effects Cr(VI) addition to indigenous microbial communities.

After 30 days incubation of the soils with increasing concentrations of Cr(VI), chemical analysis were performed and they showed that the adsorption models had underestimated the adsorption capacity of soils and that better models were needed to describe Cr(VI) adsorption in soils. For this experiment they were satisfactory, as they were just needed to compare the differences in Cr(VI) adsorption capacities between soils.

Chemical analyses also showed that the addition of Cr(VI) had a different effect in the pH of the three soils. Arnhall and Glencorse had a significant increase in pH with Cr(VI) addition, while the change in Aldroughty was not that evident (Aldroughty had a higher buffering capacity).

The addition of Cr(VI) to soils had significant effects on the concentrations of Ca, Mg and S in soil pore waters. It was also found that S contributed significantly to soil pore water pH.

Biological analyses showed that the toxicity of Cr in soils with different physicochemical properties varied depending on the type of bioassay employed. The *E. coli* pUCD607 bioassay and the root growth assay responded to Cr(VI) addition in soils in a different way to the indigenous soil microbial communities (according to PLFAs and CLPP).

When soils were analysed using *E. coli* pUCD607, the toxicity of Cr(VI) in the different soils increased with decreasing adsorption capacity, *i.e.* the toxicity decreased in the order Aldroughty (EC_{25}= 31mg/kg) > Glencorse (EC_{25}= 55 mg/kg) > Arnhall (EC_{25}=110 mg/kg). In comparison, Chaudri *et al.* (2000), using a Rhizobium-based luminescent biosensor, found that the EC_{25} value for Zn added to sandy loam soil was 164 mg/kg.

Chromium (VI) added and Cr(VI) in soil pore waters seemed to be the most important factors controlling toxicity of soils for *E. coli* pUCD607. The toxicity of Cr(VI) added to soils where *Hordeum vulgare* seedlings were growing showed a similar trend, with the soil with lowest adsorption capacity having the highest toxicity (Aldroughty). EC_{25} values for Aldroughty and Arnhall were 24 and 98 mg/kg, respectively, slightly lower than those calculated for *E. coli* pUCD607.

On the other hand, when analysing the community structure using PLFAs, the toxic response of the soils seemed different. The overall structure of the soil community (represented by the PLFAs analysis) in Aldroughty did not change significantly with the addition of Cr(VI).

Arnhall seemed to be the most toxic soil and the effect of pH was very marked.

A difference in microbial biomass was observed between contaminated and non-contaminated soils; a similar effect has been reported when heavy metals from sewage sludge were added to soil (Chander and Brookes, 1991).

According to microbial biomass (total PLFAs), the toxicity of Cr(VI)-contaminated soils seemed to follow the order Arnhall (EC_{25}=47 mg/L) > Glencorse (EC_{25}=88 mg/L) > Aldroughty (no EC available) and according to Table 5.11, the most sensitive microbial guild was Gram-negative bacteria (EC_{25}=29 mg/kg in Arnhall, EC_{25}=56 mg/kg in Glencorse, EC_{25} not available for Aldroughty) and the least sensitive was fungi (EC_{25} =348 mg/kg in Arnhall, EC_{25}= 213 mg/kg in Glencorse, EC_{25} not available for Aldroughty).

It was also found that Gram-negative bacteria were more abundant than Gram-positive bacteria in the three soils. Gram-negative bacteria have been reported to dominate in metal-contaminated soils compared with Gram-positive bacteria (Hiroki, 1992; Wenderoth and Weber, 1999), but there also exist reports where Gram-positive bacteria in metal polluted soils dominate (Roane and Kellog, 1996) The ratio of Gram-positive to Gram-negative changed slightly in Arnhall and Glencorse towards more Gram-positive bacteria with the addition of Cr(VI). An increase of Gram-positive bacteria with increase in heavy metal pollution has been reported previously (Pennanen, 2001).

It was also observed that Cr(VI) was less toxic to fungi and the eukaryotic guilds, which is in agreement with other studies, where fungi appeared to be more tolerant to heavy metals than bacteria

(Nordgren *et al.*, 1986; Hiroki, 1992). Some authors have found that in ecto- and endomycorrhizal fungi, heavy metals were bound to cell wall components such as chitin, cellulose derivatives and melanin (Galli *et al.*, 1994). The rate at which Cr enters the cell would be decreased by the accumulation in the cell wall, which would explain the lower toxicity observed.

The CLPP dye test experiment showed that Aldroughty had the lowest respiration rate (basal respiration) of the non-amended soils (Figure 5.39). Which agreed with the findings of PLFAs in non-amended soils, where Aldroughty had the lowest microbial biomass (Figure 5.28).

The results obtained from the dye test also supported the results obtained on the effect of Cr(VI) added to the community structure Arnhall in general had the lowest metabolic capacity (Figure 5.44), followed by Glencorse. It is interesting to note that Aldroughty contaminated soils had a much higher metabolic capacity than the other two soils and that activity was particularly high for the consumption of cysteine, an amino acid containing thiol groups (reduced sulphur). The utilisation of cysteine in the non-contaminated soil was not as high as in the Cr(VI)-contaminated Aldroughty soils, probably indicating a shift in the contaminated soils towards microorganisms that can utilise S (higher concentrations of S in Aldroughty were found in the soil pore waters related to Cr(VI) addition).

It has been reported in the literature that Cr(VI) can enhance the growth and sulphate-reducing activity of sulphate-reducing bacteria (Karnachuk, 1995). Sulphate reducing bacteria can conserve energy by the dissimilatory reduction of SO_4^{2-} and a spore-forming reducing bacteria has also been found to grow with Cr(VI) in addition to

various sulphur compounds as electron acceptors (Tebo and Obraztsova, 1998). If sulphate reducing bacteria could grow in Aldroughty, they would probably be able to detoxify some of the Cr(VI) by reducing it to Cr(III) (which could precipitate as $Cr(OH)_3$) (Lloyd *et al.*, 2001).

The protection from the smaller fluctuations in pH from treatment to treatment in Aldroughty and the possibility of the presence of sulphate reducing bacteria in higher amounts than in the other two soils, could also explain why a different toxicity is recorded for Aldroughty in acute (*E. coli* pUCD607 and root growth) and chronic assays (microbial community analysis and CLPP). This would merit further investigation.

The EC values obtained from all bioassays indicated that the healthy development and functionality of soil communities and plants growing in Cr-contaminated soils can be impaired at relatively low levels of Cr(VI), for example levels of Cr-contamination found in the samples collected from the field (Chapter 4) might result in serious damage to the indigenous microbial communities.

In the United Kingdom, trigger concentrations of Cr(VI) in allotments and open space are 25mg/kg, and to protect the soil quality, the maximum addition of Cr in sewage sludge should be 400 mg/kg. Assuming that a maximum 10% of total Cr could be found as Cr(VI), these values could result in the damage of some microbial communities and sensitive plants. Therefore, a revision on current Cr(VI) limits may be needed. Rüdel *et al.* (2001) have also suggested the revision of precautionary levels for Cr(VI) in the German Federal Soil Protection and Contaminated Sites Ordinance, as they found the current limits (3-10 mg Cr(VI)/kg) can result in damage of some

species. They reported EC_{50} values a low as 1 mg/kg using the ammonium oxidase activity assay.

5.9 Conclusions

The main findings of this experiment can be summarised, as follows:

- The adsorption of Cr(VI) onto soils is not accurately described by a two surface Langmuir equation, so a more complex model might be necessary in order to include factors that might be responsible for the adsorption, such as surface protonation.

- The conditions under which adsorption isotherms were run and the conditions for the microcosm experiment were different and, therefore, influenced the amount of Cr(VI) sorbed/precipitated on surfaces. In general, Cr(VI) concentrations in soil pore waters were lower than those predicted by the models.

- Possible reduction of Cr(VI) to Cr(III) and precipitation of the latter could account for some of the differences between modelled and experimental data.

- The adsorption of Cr(VI) onto soils, according to the experimental results, decreased in the order Arnhall > Glencorse > Aldroughty, the same order in which organic matter content decreased.

- Organic matter content might contribute to the toxic effects of Cr(VI) on microorganisms and plants by means of chemical

interactions with Cr(VI) (binding/scavenging) and/or biological effects on the metabolism of the microbial cells, or effects on community composition.

- Acute (single species bioassays) and chronic toxicity (microbial community analysis) of Cr(VI) gave different results.

- The toxicity of Cr(VI) in the soils to *E. coli* pUCD607 and *Hordeum vulgare* seedlings increased with decreasing Cr(VI) adsorption capacity, *i.e.* the most toxic soil was Aldroughty and the least toxic Arnhall.

- Different types of microorganisms are present in the different soils and it is possible that this makes them inherently more sensitive/resistant to Cr(VI).

- The structure of the soil community represented by the PLFA analysis did not change significantly with the addition of Cr(VI) to Aldroughty. Arnhall seemed to exhibit more Cr toxicity than Glencorse and the effect of pH was very marked in this last soil. The toxicity of soils in this case seemed to follow the order Arnhall > Glencorse > Aldroughty.

- The most sensitive guild was Gram-negative bacteria and the least sensitive guild was fungi.

- Findings from the dye test experiment, on the metabolic capacity of the three soils, support the results from the PLFA analysis, with Aldroughty having the highest metabolic capacity and recording a high activity for microorganisms that can utilise cysteine.

- The possibility of Aldroughty being better able to support sulphate reducing bacteria and the fact that pH changes between treatments were not as marked as in the other two soils, could explain the difference in recorded toxicity between acute and chronic toxicity assays.

- According to the EC values obtained for the different PLFA guilds, the levels of Cr(VI) that have been found in some samples from the chromium contaminated sites discussed in Chapter 4 (up to 4.2 g Cr(VI)/kg soil and "available" Cr(VI) up to 700 mg/kg), could damage the indigenous microbial communities.

- The revision of current regulatory limits is advised in order to avoid damage to soil functionality.

- In this experiment, the single species bioassay did not seem to represent what happened to the soil microbial community, although it can be considered an indicator of acute toxicity, *e.g.* for the leaching of soils, and could, therefore, be used as an indicator to compare potential toxicity between different systems.

- It is important to recognise that the different assays give different pieces of information. While the single species bioassays can be good indicators of acute toxicity, community analysis accounts for the complex chemical and biological interactions which develop over time and which result in the bioavailability and chronic toxicity of a contaminant.

Chapter 6

Pot Experiment

6.1 Introduction

In the previous experiment, the toxicity of Cr(VI) to indigenous soil microorganisms was tested in soils with different physicochemical properties and Cr(VI) adsorption capacities. The microcosm used was, to a certain degree, a very simple one - Cr(VI) was introduced in a synthetic solution and microorganisms were not provided with any kind of external nutrient input, relying purely in the organic matter available in the soils. Nevertheless, in natural systems, other organisms might be present in soils and the interaction between different chemical and biological processes can change the conditions and resistance or sensitivity of microorganisms to contaminants. In a simple system like the microcosm in Chapter 5, the acute toxicity of chromium contaminated soils could be predicted from the knowledge of the adsorption capacity of the soil and simple acute tests were highly correlated with Cr(VI) in solution. The chronic toxicity of Cr(VI), on the other hand, was the result of more complex biological and chemical processes in the soils. Still, it was important to study the effects of Cr(VI) addition to soil in more complex and closer to natural systems, like systems including plant-soil interactions. The symbiotic and complex mechanisms in soil-plant-microorganism systems can also lead to very complex relationships between toxicants and these systems. Plants may modify the soil environment affecting the bioavailability of Cr and are also a source of C and energy for microorganisms.

One of the potential problems associated with the COPR contaminated sites is that the leachate could potentially reach non-contaminated soils and affect existing soil-plant-microorganism systems. The leachate from COPR-contaminated soils is a more complex solution than a synthetic Cr(VI) solution, therefore it could

have different effects on soil properties and soil microbial communities.

In order to test the toxicity of Cr(VI) from COPR leachate in more complex systems, two clean soils, studied in the previous experiments, with different Cr(VI) adsorption capacities were selected. Seedlings of *Hordeum vulgare* (barley) were planted in a series of pots, left to establish and treated with solutions either free of Cr or containing Cr for a period of time, after which plants were harvested and analysed. Soil physicochemical properties were analysed and the structure and metabolic capacity of microbial communities was studied by phospholipid fatty acids and community level physiological profiles, respectively.

6.2 Objectives and hypotheses

In sites that contain COPR material, continuous leaching might occur as a result of rainfall or flooding, with the possibility of reaching nearby soils. Consequently this experiment studied the following effects:

- The effects of COPR leachate contaminating soils in which plants could be growing, including the effects on soil indigenous microorganisms, plants and soil physicochemical characteristics.

- The effects of COPR leachate compared to the effects of synthetic Cr(VI) solutions and solutions of the same composition as the leachate but without Cr.

- The effects of the leachate on soil microorganisms and plants when the leachate reaches two soils with different Cr(VI) adsorption capacities.

The main hypotheses tested in this experiment were:

- COPR leachate will affect plant growth by altering the soil physicochemical properties (especially pH).

- COPR leachate will lead to an accumulation of Cr in soils and plants and result in toxicity to both plants and microorganisms.

- Cr(VI) will be the major contributing factor to reduced plant growth.

- The toxicity of COPR leachate will be higher in soils with a lower Cr(VI) adsorption even in the presence of plants.

- The toxicity of Cr(VI) will be similar regardless of its form (COPR leachate or synthetic soil solution).

- Cr(VI) toxic effects will be different in systems where plants are growing, compared with systems where there are no plants.

- The metabolic capacity of microbial populations will be affected by Cr(VI).

6.3 Experimental overview

Aldroughty and Arnhall soils were used to plant seedlings of *Hordeum vulgare*. These soils, which had been previously studied, had different physicochemical properties and Cr(VI) adsorption capacities (Section 5.3).

Plants were left to establish and then four different treatments were applied (using 4 replicates). Solutions used as treatments consisted of deionised water; COPR leachate; a solution similar in elemental composition to the COPR leachate but without Cr; and a synthetic Cr(VI) solution with the same Cr(VI) concentration as the COPR leachate. Plants were watered with the same volumes of treatment solutions for 20 days (total concentration of Cr(VI) equalled 50 mg/kg), after which, plants were harvested and chemical and biological analyses were performed on the soil samples.

6.4 Experiment set-up

Aldroughty and Arnhall were used in this experiment, as their properties and Cr(VI) adsorption capacities were quite different and had been studied previously (Sections 5.2 and 5.3).

6.4.1 Plant germination

Barley seeds (*Hordeum vulgare*) were placed in a dessicator with deionised water and connected to a vacuum line. The vacuum was opened for a few minutes to allow the seeds to incorporate the water. Once the seeds were imbibed, excess water was eliminated and the seeds were placed over paper towel saturated with deionised water

and kept in a dark place at room temperature (20 °C). The seeds germinated in approximately 24 hours.

6.4.2 Potting and plant establishment

Six seedlings with stems of approximately 1 cm in length were planted in 10 cm plastic pots containing 300 g of soil.

In total, 20 pots were planted for each soil. The soils were watered to 70% of their water holding capacity (calculated as described in Section 2.1.2) and placed in a controlled environment cabinet (Control Environments LTS (CONVIRON®) Winnipeg, Canada) under the following conditions:

- Day length: 14 hours, including 1 hour to increase and decrease the light intensity (sunrise and sunset).

- Initial temperature: 20°C during the day and 17°C during the night with a ramping of 1.5 hours at the beginning and the end of the day light period.

- Relative humidity: 70%.

- Light input: light was provided using a combination of equal amounts of a metal halide and a high-pressure sodium lamp, providing a photosynthetic radiation of 500-550 $mm^{-2}s^{-1}$ at plant level.

Plants were watered regularly when the light intensity and temperature were low, to avoid damaging them. The moisture in soils was maintained at 70% of the soil water holding capacity.

By the 5th day, the roots started to outgrow of the pots, and plants had to be transplanted into bigger pots. They were transplanted to pots that contained in total 990 g and 970 g of Aldroughty and Arnhall soils, respectively, including all the soil that had been originally used in the smaller pots.

To slow plant growth, the temperature was decreased to 17° C during the day and 15° C during the night.

Cereals have distinctive growth stages that represent different physiological status for the plants. These can be divided into tillering, stem elongation, flowering, ear emergence and ripening (Agricultural Press, 1978). Originally it was planned to have the same number of tillers in plants in both soils before applying the treatments, but the different rate at which plants grew did not allow for this, therefore the plants were harvested at the same time independently of their tiller number. Plants were harvested during the tillering/stem prolongation phase.

6.4.3 Preparation of treatments

Control treatment. This consisted of deionised water, pH 5.5.

COPR leachate. The material collected from the COPR-contaminated site (Chapter 4), with the highest Cr(VI) concentration, was used to obtain the leachate (sample labelled as 5). A solution of 1 mM NaCl solution was passed through COPR-soil material (< 2mm) that had been packed inside a Perspex column. The NaCl solution was pumped using a peristaltic pump, keeping the flow rate constant at 8.3 ml/h. In order to keep the concentration of the collected fractions at around 1.0 mM Cr(VI), the COPR material in the

columns was replaced with fresh material periodically. This procedure was repeated several times until enough leachate was obtained for the experiment. The leachate presented a strong yellow colour (Figure 6.1 a and b). The collected fractions were mixed, homogenised and analysed for Cr(VI) by the spectrophotometric method and for soluble elements by ICP-OES (Sections 2.2.5 and 2.2.6). The leachate had a pH of 12.08 and its major components were Cr(VI) (1.04 mM), Ca (4 mM) and S(0.3 mM).

Synthetic leachate. A solution with a "similar" composition to that of the leachate but without Cr(VI) was prepared using 3.7 mmol $Ca(OH)_2$, 0.3 mmol $CaSO_4$ and 100 mmol NaOH in 1 L of deionised water. The solution was stirred continuously, although complete dissolution was not achieved, probably due to the incorporation of CO_2 into the solution and precipitation of Ca as $CaCO_3$. The solution was shaken well before for watering the plants.

Synthetic Cr(VI) solution. A solution containing 1.04 mM Cr(VI) was prepared from K_2CrO_4. The pH of the resulting solution was 9.5.

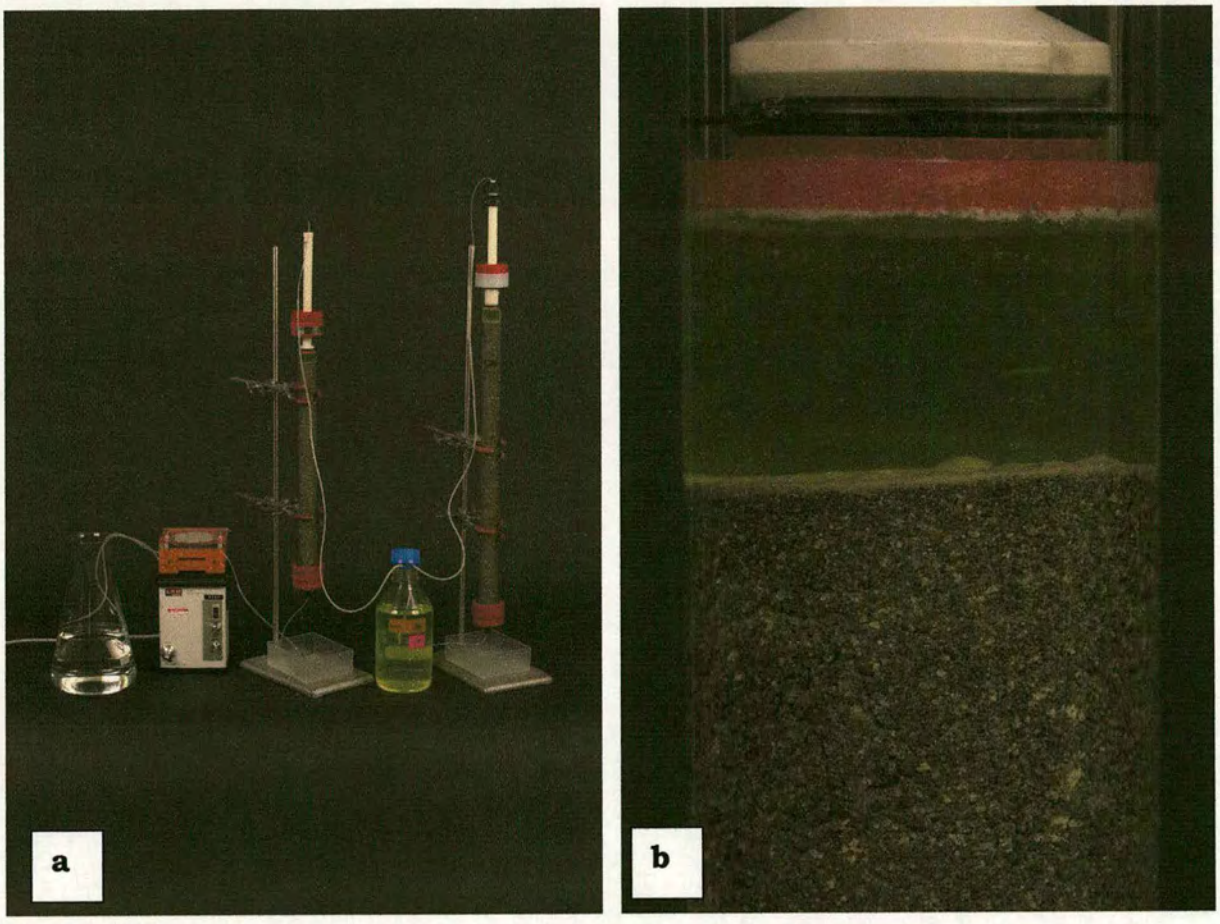


Fig. 6.1 a) Leachate extraction columns and b) a close up of extract of COPR material (sample 5).

6.4.4 Treatment application

The application of the treatments started when the plants had between 4 and 6 leaves unfolded. The plants already had between 1 and 3 tillers at this stage.

The pots for each soil were divided at random (using a table of random numbers) into 4 groups and labelled as control (deionised water), leachate (COPR leachate), synthetic leachate (no Cr) and synthetic Cr(VI) solution. Four replicates were used for each treatment.

During the first 10 days, plants were watered every day with 50 ml of the treatment (increased to 100 ml by the end of the experiment); the rest of the liquid that was needed to keep the moisture constant was added as water. The total amount of Cr(VI) added was approximately 50 mg per kg of dry soil.

By the end of the experiment the plants absorbed different quantities of water in the two soils so it was not possible to maintain the moisture as consistently as before. Plants growing in Arnhall transpired water at a slower rate than plants growing in Aldroughty.

6.4.5 Harvesting

The plants were harvested after 20 days, separated into roots and foliage and dried. Soils were weighed and placed into polyethylene bags and stored at 4°C. Sub-samples of soils were immediately frozen at –25°C and then freeze-dried to use in the PLFA analysis.

6.5 Experiment results

6.5.1 Observations during seed germination and plant establishment

The method used to artificially imbibe the seeds allowed for fast seed germination, without apparent damage to the seeds, as in general, seeds germinated more or less at the same time and few were non-viable.

The stem growth was very fast and for the first few days after planting, seedlings in Aldroughty developed quicker than those in Arnhall. Nevertheless, after 5 days, the growth rate had changed, stems in Aldroughty had stopped their quick development and stems in Arnhall were taller and thicker (10 cm height). The length of stems growing in Arnhall also seemed more homogenous than in Aldroughty.

After two weeks the leaves of plants growing in Aldroughty developed yellow stripes, while plants in Arnhall remained green. Aldroughty, with a neutral pH, might have been retaining micronutrients strongly and causing nutrient deficiencies in the plants, therefore a nutrient solution (Table 6.1) was added to all the plants (0.11 ml per g dried soil) to allow for recovery before initiating the experimental treatments After the application of the nutrient solution, plants seemed to recover and new leaves were greener.

Table 6.1 Nutrient solution used before starting treatments.

Salt	Concentration (mg /L)	Salt	Concentration (mg/L)
$(NH_4)_2 \cdot SO_4$	236	$MgSO_4 \cdot 7H_2O$	20.3
K_2SO_4	61	$MnCl_2 \cdot 4H_2O$	0.72
$CuSO_4 \cdot 5H_2O$	0.12	$NaMoO_4 \cdot 2H_2O$	0.01
$FeCl_3 \cdot 6H_2O$	1.7	H_3BO_3	0.57
$NaH_2PO_4 \cdot 2H_2O$	36	$ZnSO_4 \cdot 7H_2O$	0.13
$CaCl_2$	10.9		

6.5.2 Soil analyses

6.5.2.1 Soil water content

Immediately after the harvest, samples of the soils were taken and their moisture measured as described in Section 2.1.1. Figure 6.2 shows the moisture content of the harvested soils.

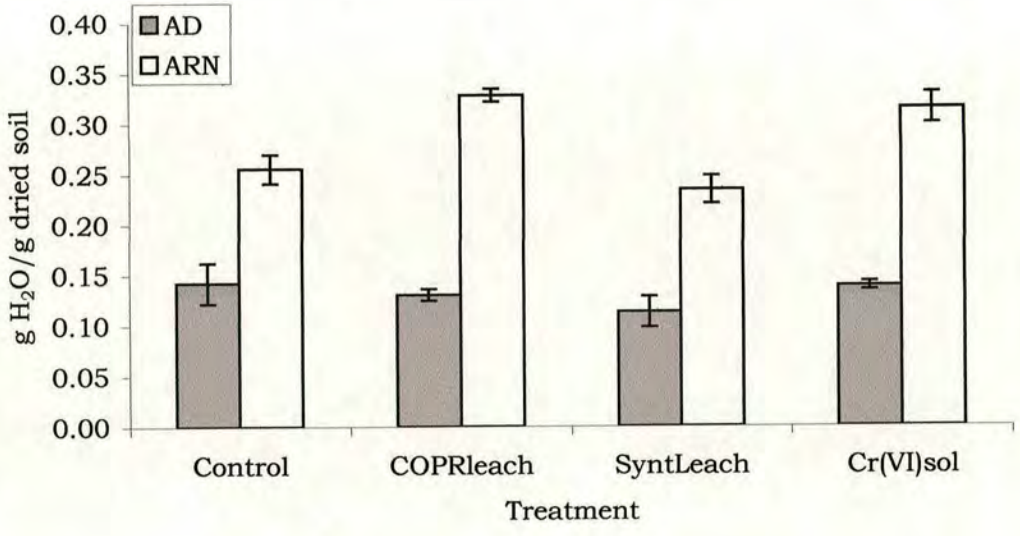


Fig 6.2 Soil water content of Aldroughty (AD) and Arnhall (ARN) soils different treatments. Control = deionised water, Syntleach = Synthetic Leachate, COPRleach = COPR leachate, Cr(VI)sol = Cr(VI) synthetic solution.

The analysis of variance of soil moisture data, showed that there was a significant difference between soils ($P<0.001$), that the effect of

treatments in Aldroughty was not significant (P= 0.442), but that the effect of treatments in Arnhall was highly significant (P<0.001).

6.5.2.2 Soil pH

The treatments had a highly significant effect on soil pH (P<0.001). In both soils the largest change in pH was observed in soils treated with the synthetic leachate, followed by the soils treated with the COPR leachate (Figure 6.3). The largest change in pH was recorded in Aldroughty, between 0.2 and 0.5 pH units.

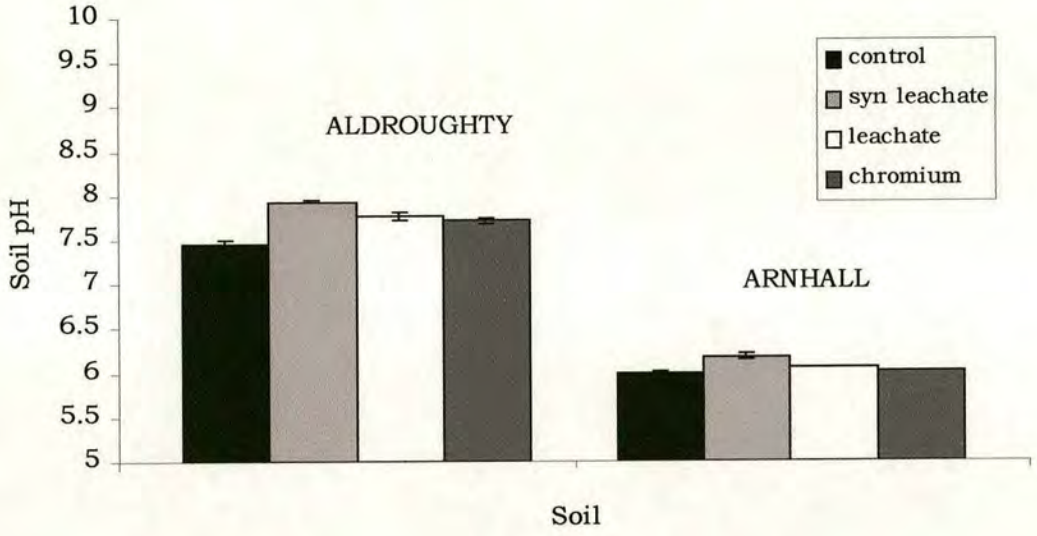


Fig. 6.3 pH values of Aldroughty and Arnhall soils after harvesting.

6.5.2.3 Soil chemical composition

In general, Arnhall soils presented a higher concentration of major and minor elements than Aldroughty. In the experiments described in Chapter 4, it was found that the Cr(VI) adsorption capacity of Aldroughty was very low compared to that of Arnhall. In this experiment, the amount of Cr(VI) adsorbed by Aldroughty was lower than that of Arnhall.

The chemical compositions of Arnhall and Aldroughty determined from the *aqua regia* digestions of soils (Section 2.2.2) are depicted in Figures 6.4 to 6.6. The analysis of variance of individual elements showed that the addition of the treatments to the soils had significant effects on Al ($P = 0.098$), B ($P<0.001$), Cr ($P<0.001$), K ($P=0.013$), Mn ($P<0.001$), Na ($P<0.001$), Se ($P<0.001$) and Zn ($P=0.075$). The COPR leachate and synthetic Cr solution resulted in the same levels of Cr contamination in both soils, which were 50 mg per kg of dried soil.

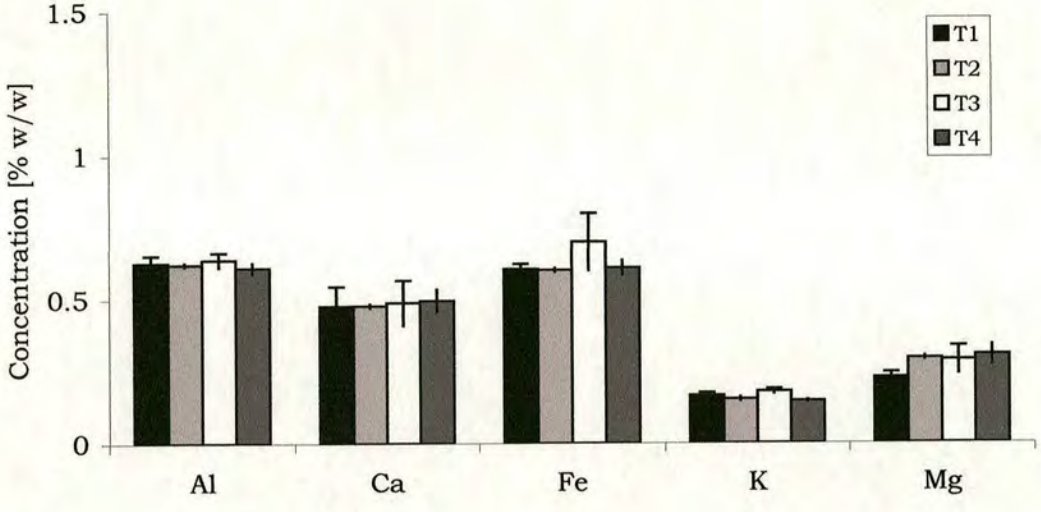


Fig. 6.4 Concentration of major elements in Aldroughty after harvest (obtained by *aqua regia* digestions). T=1 control; T2=Synthetic Leachate; T3= COPR Leachate; T4= Cr(VI) synthetic solution.

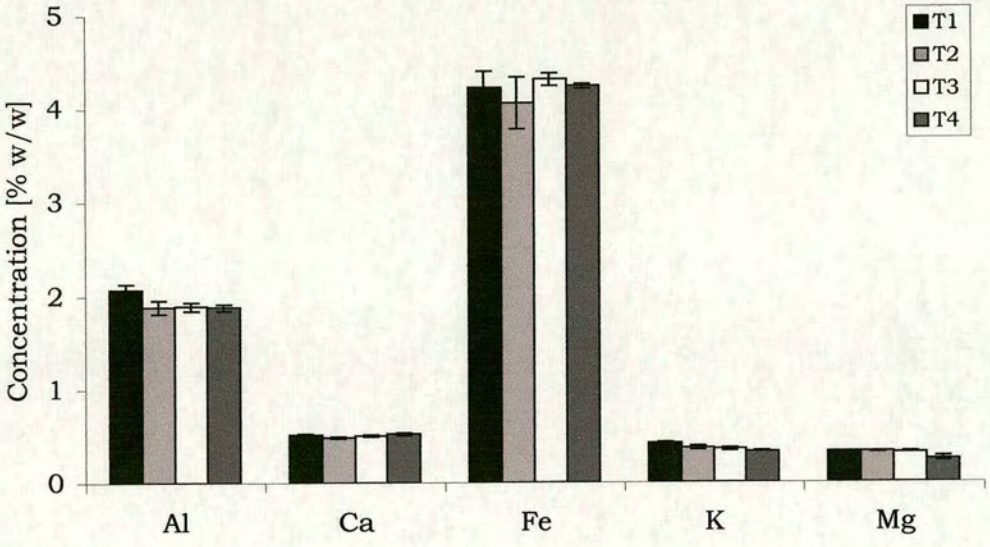


Figure 6.5 Concentration of major elements in Arnhall after harvest (obtained by *aqua regia* digestions). T=1 control; T2=Synthetic Leachate; T3= COPR Leachate; T4= Cr(VI) synthetic solution.

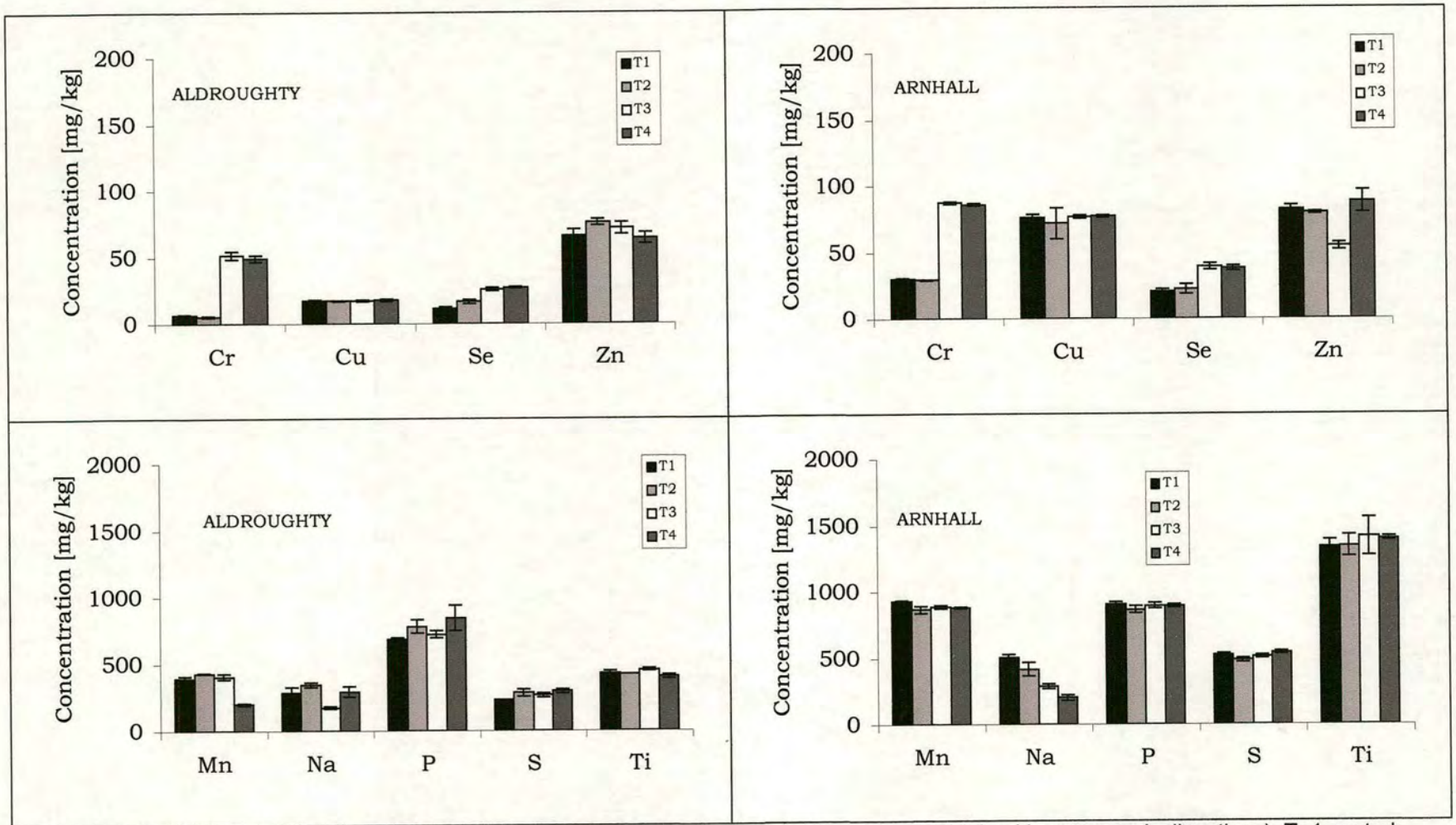


Fig. 6.6 Concentration of other elements in Aldroughty and Arnhall soil samples after harvest (obtained by *aqua regia* digestions). T=1 control; T2=Synthetic Leachate; T3= COPR Leachate; T4= Cr(VI) synthetic solution

6.5.2.4 Extractible Cr(VI)

Sub-samples of harvested soils were taken to 50% of their water holding capacity, stored for a week and then centrifuged to extract the soil solution as described in Section 2.2.1. A small volume of soil solution was collected and analysed by ICP to determine its elemental composition. Results of the analysis were highly variable and were not considered appropriate to compare the effect of Cr(VI) in solution to *E. coli* puCD607. Instead, Cr(VI) extracted by phosphate buffer was used to consider "available" Cr(VI).

Soil sub-samples were used to extract exchangeable Cr(VI) using the phosphate extraction method described in Section 2.2.4. The resulting extracts were analysed for Cr(VI) using the diphenylcarbazide method described in Section 2.2.5. The concentration of exchangeable Cr(VI) was higher in Arnhall than in Aldroughty (Figure 6.7),

Both in Arnhall and Aldroughty, the exchangeable Cr(VI) extracted was higher in soils that had been treated with the COPR leachate than in soils that had been treated with the synthetic Cr(VI) solution.

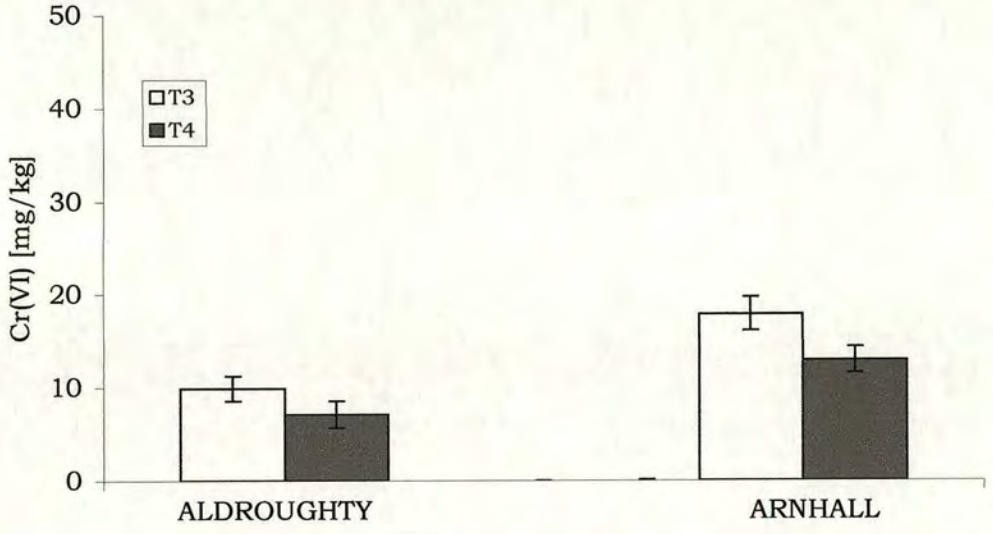


Fig. 6.7 Chromium (VI) in phosphate extracts from Aldroughty and Arnhall soils treated with Cr(VI), either as COPR Leachate (T3) or Cr(VI) synthetic solution (T4)

6.5.3 Effects of treatments on plants

6.5.3.1 Observations from harvesting

When the plants were harvested it was noticed that the roots of Aldroughty controls and synthetic leachate had colonised most of the soil, the roots in the other two treatments were smaller, less abundant and in general without secondary roots. The roots in the controls and synthetic leachate treatments of Arnhall were less abundant but thicker than those in Aldroughty. The fresh weight of roots changed significantly (P=0.016) with the treatment (Figure 6.8).

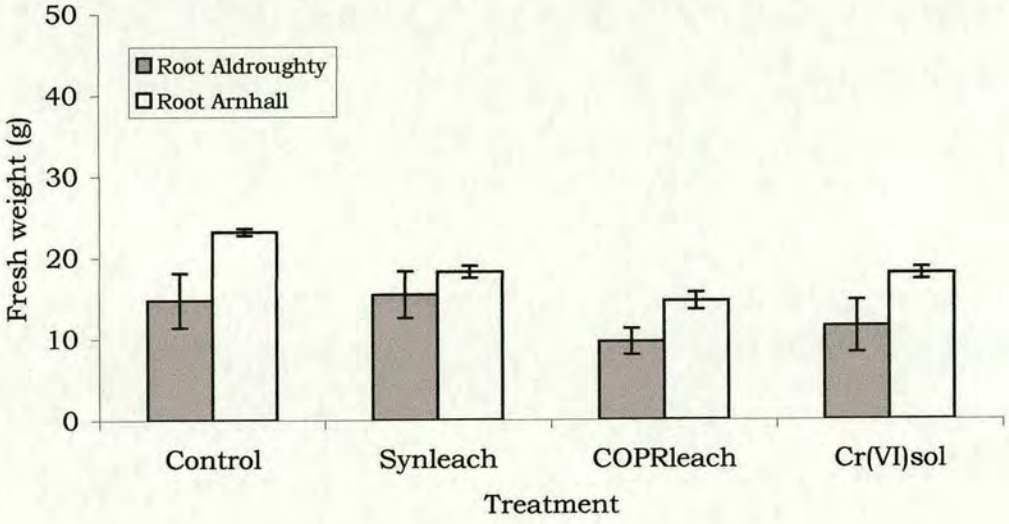


Fig. 6.8 Fresh root weight of barley plants grown in two soils watered with four different treatments. Control = deionised water, Synleach = Synthetic Leachate, COPRleach = COPR leachate, Cr(VI)sol = Cr(VI) synthetic solution.

The lowest weight was recorded for the COPR leachate treatments in both soils. In general the weight of fresh roots was lower for Aldroughty than for Arnhall. The opposite occurred with respect to shoot production (Figure 6.9). Arnhall soils produced plants with more leaf biomass than Aldroughty soils and the leaves seemed to be affected in a similar way by the leachate and Cr(VI) treatment. The plants had lower leaf biomass weight for these two treatments in both soils compared with their respective controls. Compared with the controls, the synthetic leachate treatments in both soils resulted in lower root weights, but similar leaf biomass weights.

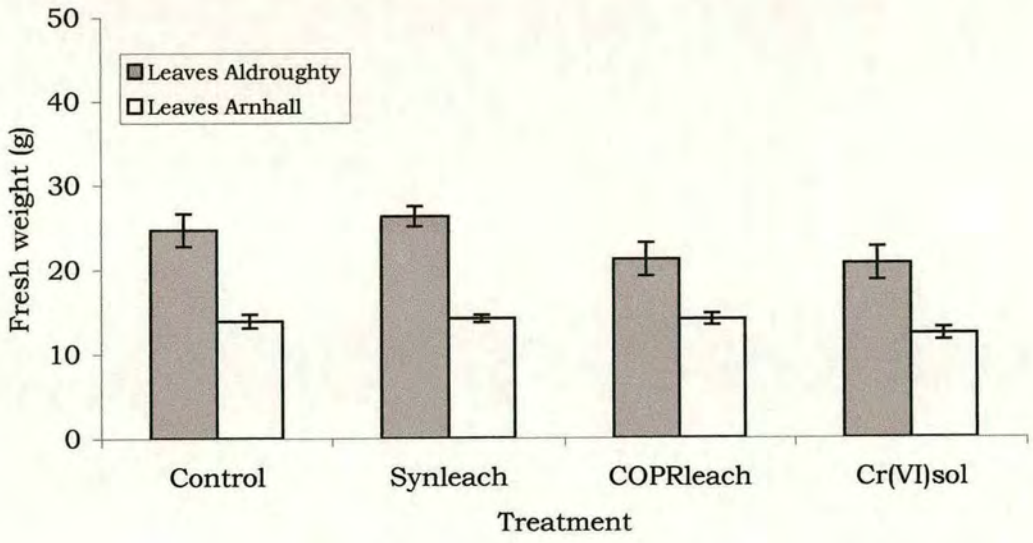


Fig 6.9. Fresh leave biomass weight of barley plants grown in two soils watered with four different treatments. Control = deionised water, Synleach = Synthetic Leachate, COPRleach = COPR leachate, Cr(VI)sol = Cr(VI) synthetic solution.

The analysis of variance for fresh root and leaves weight, using treatment as factor and blocked by soils, showed that the type of soil and treatment had a significant effect on root weight (P= 0.016) and on leaf weight (P= 0.052).

6.5.3.2 Elemental composition of plant material

The plant material was ashed (Section 2.1.4) and digested using *aqua regia* (Section 2.2.2). The major elements in the root and leaves are shown in Tables 6.2 and 6.3.

Table 6.2 Chemical composition of leaves from plants grown in Aldroughty and Arnhall and watered with four different treatments.

Soil	Treatment*	Element						
		Al	**Ca**	**Cr**	**Cu**	**Fe**	**K**	**Mg**
		mg/kg	% (w/w)	mg/kg	mg/kg	mg/kg	% (w/w)	% (w/w)
Aldroughty	1	182	2.3	0.0	3.9	220	32	3.45
Aldroughty	2	145	2.4	0.0	2.8	157	30	3.03
Aldroughty	3	75	2.1	70.1	2.2	88	18	2.52
Aldroughty	4	98	2.0	70.3	2.0	108	23	2.89
Arnhall	1	229	4.9	0.0	3.8	388	12	3.37
Arnhall	2	134	4.5	0.7	2.8	256	13	3.06
Arnhall	3	222	4.5	33.0	2.0	409	3	2.61
Arnhall	4	267	4.4	33.3	2.3	447	6	3.01
		Mn	**Na**	**P**	**S**	**Se**	**Ti**	**Zn**
		mg/kg	% (w/w)	% (w/w)	% (w/w)	mg/kg	mg/kg	mg/kg
Aldroughty	1	21.6	24	1	2	0.7	9.7	24.7
Aldroughty	2	16.8	28	1	2	0.8	8.6	22.5
Aldroughty	3	7.5	38	2	2	22.6	4.7	19.6
Aldroughty	4	10.9	29	1	2	23.6	5.5	16.8
Arnhall	1	24.2	34	1	2	2.8	11.9	12.5
Arnhall	2	20.6	38	2	2	3.0	7.6	10.1
Arnhall	3	10.2	53	2	1	12.7	11.2	7.6
Arnhall	4	9.9	44	2	1	12.3	11.1	8.3

* Treatment: 1 = Control; 2 = Synthetic leachate; 3 = COPR leachate; 4 = Cr(VI) synthetic solution.

Table 6.3 Chemical composition of roots from plants grown in Aldroughty and Arnhall and watered with four different treatments.

Soil	Treatment	Element						
		Al	**Ca**	**Cr**	**Cu**	**Fe**	**K**	**Mg**
		% (w/w)	% (w/w)	mg/kg	mg/kg	mg/kg	% (w/w)	% (w/w)
Aldroughty	1	0.16	0.59	9.79	16.4	0.17	0.64	0.18
Aldroughty	2	0.19	0.60	4.72	13.8	0.11	0.42	0.17
Aldroughty	3	0.17	0.61	1433	15.3	0.29	0.48	0.12
Aldroughty	4	0.20	0.67	1486	15.6	0.33	0.61	0.15
Arnhall	1	0.30	0.26	4.61	18.8	0.46	0.32	0.13
Arnhall	2	0.21	0.34	8.85	13.5	0.46	0.45	0.13
Arnhall	3	0.20	0.36	764	11.2	0.32	0.39	0.10
Arnhall	4	0.18	0.35	723	11.5	0.06	0.48	0.08
		Mn	**Na**	**P**	**S**	**Ti**	**Zn**	
		mg/kg	% (w/w)	% (w/w)	mg/kg	mg/kg	mg/kg	
Aldroughty	1	75.9	0.12	0.19	619	79.1	29.3	
Aldroughty	2	39.4	0.13	0.13	517	93.6	27.4	
Aldroughty	3	64.1	0.14	0.15	652	86.8	26.8	
Aldroughty	4	66.2	0.10	0.14	698	108	32.0	
Arnhall	1	38.5	0.14	0.11	435	155	12.9	
Arnhall	2	28.5	0.26	0.11	663	123	14.9	
Arnhall	3	27.7	0.18	0.11	547	130	14.9	
Arnhall	4	56.8	0.09	0.11	476	102	14.0	

* Treatment: 1 = Control; 2 = Synthetic leachate; 3 = COPR leachate; 4 = Cr(VI) synthetic solution.

The composition of leaves had a higher variation than the composition of roots, in which Cr was found in higher concentrations. Plants growing in Aldroughty had the highest concentrations of Cr.

The difference in Cr contents in leaves between controls and treatments containing Cr(VI) was significant (P=0.065), the same was observed for the Cr content in roots (P=0.05). Aldroughty presented more Cr in the plant material compared with Arnhall. Roots seemed to have accumulated more Cr than the leaves in both soils.

The amount of Cr(VI) was not studied in the plant material, therefore it is not known if any of the Cr found by the ICP analysis had been converted to Cr(III). It would be interesting to investigate whether the plant system, possibly the roots, convert Cr(VI) into Cr(III).

The analysis of variance on the elemental composition of leaves and roots indicated that soil type and treatment had a significant effect on the concentration of Cr in leaves and roots (P<0.001), Cu in leaves (P<0.001) and roots (P=0.156), K in leaves (P<0.001), Mn in leaves (P<0.001), Na in roots (P<0.022), S in leaves (P=0.001), Se in leaves (P<0.001) and Zn in leaves (P=0.020).

To try to discard the possibility that the effects on the elemental concentrations in leaves and plants were not also the result of soil pH, the ANOVA was performed using 2 treatments, the control and the synthetic leachate, which contained no Cr(VI). Results of the ANOVA, using soil type as blocking structure, showed that the application of the treatments without Cr had an effect on the concentration of Al in leaves (P=0.086), Cu in leaves (P=0.012) and roots (P=0.101), Fe in leaves (P=0.064), Mn in roots (P=0.050), Na in roots (P=0.087) and Ti in leaves (P=0.190).

The control and the synthetic leachate differed greatly in pH, which is known to affect the uptake of several of these elements. However, there was also a difference in Ca, S and Na concentrations, therefore the effect of pH could not entirley be separated from the combined effects of these other elements.

The leachate and synthetic leachate had the same pH and main elements in the same concentrations, but the synthetic leachate had no Cr. The statistical analyses indicated that:

- there was a difference in Cr concentration in leaves and roots due to a combined effect of treatment and soil type, the effect was also shown by just treatment, but there was no effect in the leaves nor roots of plants which had not been treated with Cr;

- the application of the treatments, and particularly the application of Cr(VI) to soils had an effect on the concentrations of Mn, S and Se in leaves;

- the concentration of Al, Fe, Na and Ti in leaves was determined by the soil type and perhaps influenced by soil pH.

Visual symptoms on plants also gave an indication of stress and possibly, nutrient deficiencies. When plants were harvested, the conditions of the plants were different to those before the treatments were applied (Figure 6.10), *i.e.* plants which had been treated with Cr(VI) were smaller than the ones treated without Cr(VI). The condition of plants treated with Cr(VI) had obviously deteriorated both in Aldroughty (Figure 6.11) and Arnhall (Figure 6.12). After several days of treatment application, spots in the centre and dark

edges were clear in plants which had been treated with Cr(VI), some spots could also be distinguished and in general the colour of the leaves was of a paler green than the ones in the controls (Figure 6.13). The young leaves of plants growing in all soils tended to develop yellow tips and curled at the ends.

According to the literature, Barley under stress may show an overall leaf spotting (ADAS, 1983).

The chemical analysis of plant material also identified an effect of treatment on the content of Cu in leaves, but the effect was also found in the treatments without Cr. This might suggest that the main factor controlling Cu in the leaves was soil pH.

Figure 6.10 Plants before the treatments were applied, from left to right: two plants growing in Aldroughty, two plants growing in Arnhall.

Figure 6.11 Condition of plants growing in Aldroughty just before harvest. From left to right: control, leachate, synthetic leachate, Cr(VI) synthetic solution.

Fig. 6.12 Condition of plants growing in Arnhall just before harvest. From left to right, leachate, synthetic leachate, Cr(VI) synthetic solution, control.

Fig. 6.13 Visual symptoms of stress in experimental barley plants just before plants were harvested.

6.5.4 Effect of the treatments on the soil microbial community as studied by lipid biomarkers (phospholipid fatty acids).

Phospholipid fatty acids were extracted from freeze-dried sub-samples of soil as described in Section 2.3.3. In this case a total of 46 PLFAs with a chain length up to 20 carbons were detected by GC-MS (Table 6.4).

Table 6.4 List of PLFAs identified by GC-MS in the freeze dried samples from Arnhall and Aldroughty

12:0	16:1ω11	17:1ω8t	18:0
13:0	16:1ω7 c or t	17:1ω7	19:1ω6
14:0i	16:1ω7 t or c	17:00	18:0(10Me)
14:0	16:1ω5c	18:0br	19:1ω8
14:1ω9	16:0	17:0(10Me)	19:0cy
15:0i	17:0br	18:3 ω 6,8,13	20:4ω 6,9,12,15
15:0ai	16:0(10Me)	18:2 ω 8,12	20:5ω3
15:0	17:0i	18:2ω 6,9	20:4ω 2,6,10,14
16:0br	17:0ai	18:1ω9	20:4ω 3,6,9,12
16:1i	17:0br	18:1ω7	20:1ω9
16:1ω11	17:1ω8c	18:1ω13	20:1
16:0i	17:0cy	18:1ω10 or 11	20:0

Data obtained was subjected to principal component analysis and the resultant scores were plotted. The graphs of scores of component 2 against component 1 (Figure 6.14), showed a clear separation between the PLFA profiles of the Aldroughty and Arnhall soils, as well as a clear separation between treatments with and without Cr. Treatments 1 and 2 (control and synthetic leachate) and 3 and 4 (COPR leachate and Cr (VI) solution) were also distinguished from each other and these differences were the same for both soils. The difference between treatments was more obvious in Aldroughty.

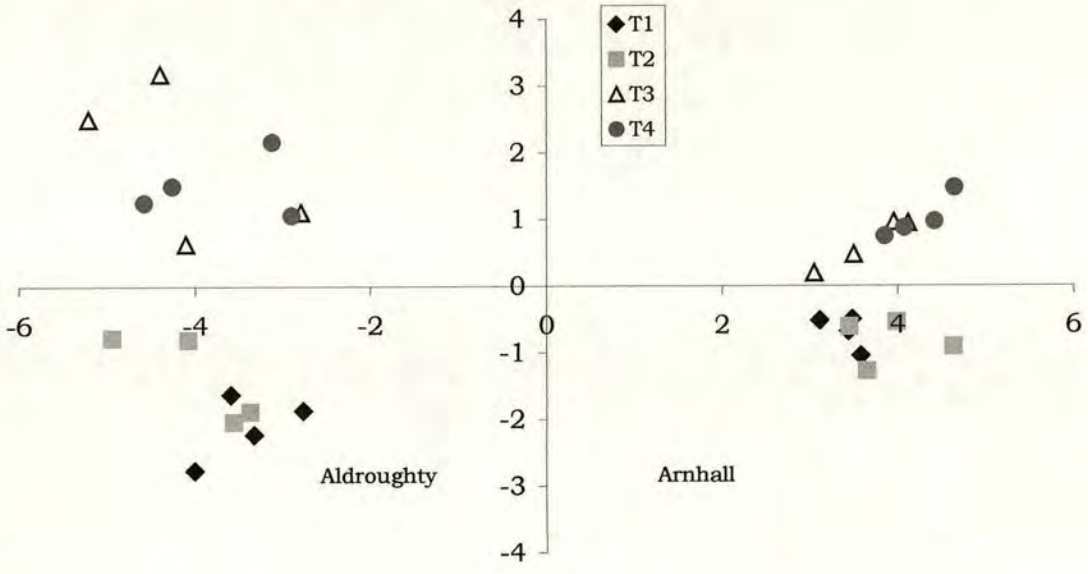


Fig. 6.14 Ordination plot of principal components scores (PC 2 *versus* PC1) for two soils treated under four different treatments: T1= control (deionised water); T2=synthetic leachate without Cr, T3=COPR leachate and T4= synthetic Cr(VI) solution.

Principal components 3 and 4 (data not shown) did not give any further resolution of the treatments.

The effect of the treatments on the PLFAs associated with different groups of organisms was compared using the percentage of PLFAs (as expressed by mol percentage of the total PLFAs for each control). Figure 6.15 shows the effects of the treatment on the total microbial PLFAs (total microbial biomass) (Pennanen *et al.*, 1998), for both Aldroughty and Arnhall. A decrease of around 30-35% in the total PLFAs was observed for the treatments containing Cr(VI) and around 5-15% for the synthetic leachate which had a higher pH. The decrease in total microbial biomass was greater in the Aldroughty soil than in the Arnhall soil. There was a highly significant effect ($P<0.001$) of treatment and type of soil on the total PLFAs. For each

soil type the treatments also had a significant (P=0.014) effect on the total PLFAs in Aldroughty and a highly significant effect on the PLFAs in Arnhall (P<0.001). The treatment that showed the highest reduction on total PLFAs in Aldroughty was the Cr(VI) synthetic solution, very closely followed by COPR leachate. In Arnhall the opposite was shown (highest reduction for the COPR leachate followed by the Cr(VI) synthetic solution).

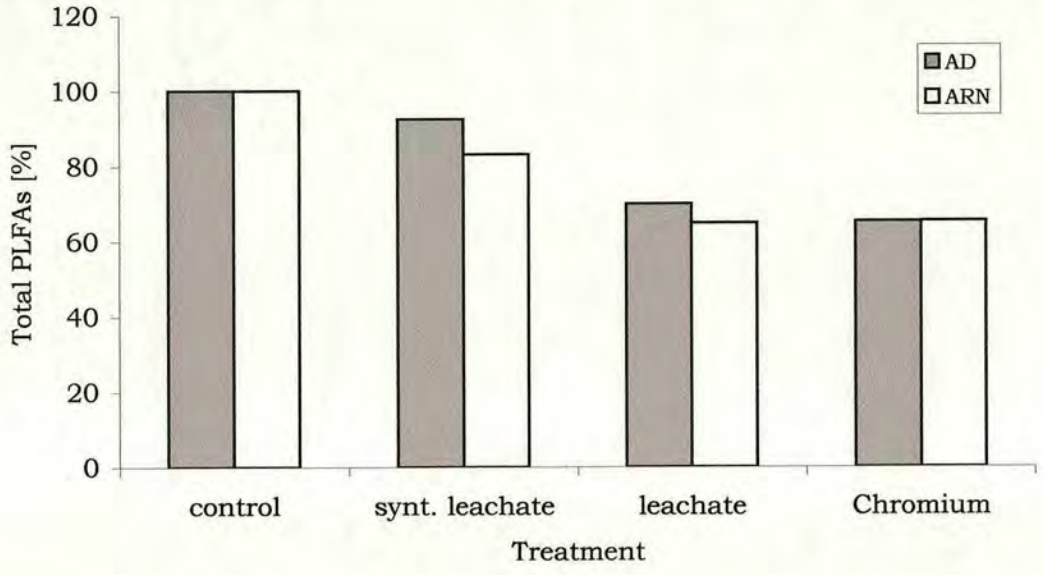


Fig. 6.15 Total PLFA content (relative to control) of two soils under four different treatments control (deionised water); synthetic leachate without Cr, COPR leachate and a synthetic and CrVI solution.

Figure 6.16 shows the effect of treatments on the bacterial PLFAs (15:0i, 15:0a, 15:0, 16:0i, 16:0, 17:0i, 17:0a, 17:0cy, 18:0ω9) (Yao *et al.*, 2000; Frostegärd *et al.*, 1993) was similar to the effect shown in the total PLFAs, with a decrease of around 30-35% in the bacterial PLFAs in the treatments containing Cr(VI) and a decrease of 5-10% in the treatments at high pH. Treatment and soil type had a highly significant (P<0.001) effect on the bacterial PLFAs. There was a significant (P=0.019) effect of treatment on the bacterial PLFAs in

Aldroughty and a highly significant (P<0.001) effect of treatment on the bacterial PLFAs in Arnhall.

The COPR leachate caused the greatest reduction in bacterial PLFAs in Arnhall closely followed by the synthetic Cr(VI) solution. The opposite was shown in Aldroughty, the highest reduction of PLFAs was shown for soils treated with Cr(VI) synthetic solution.

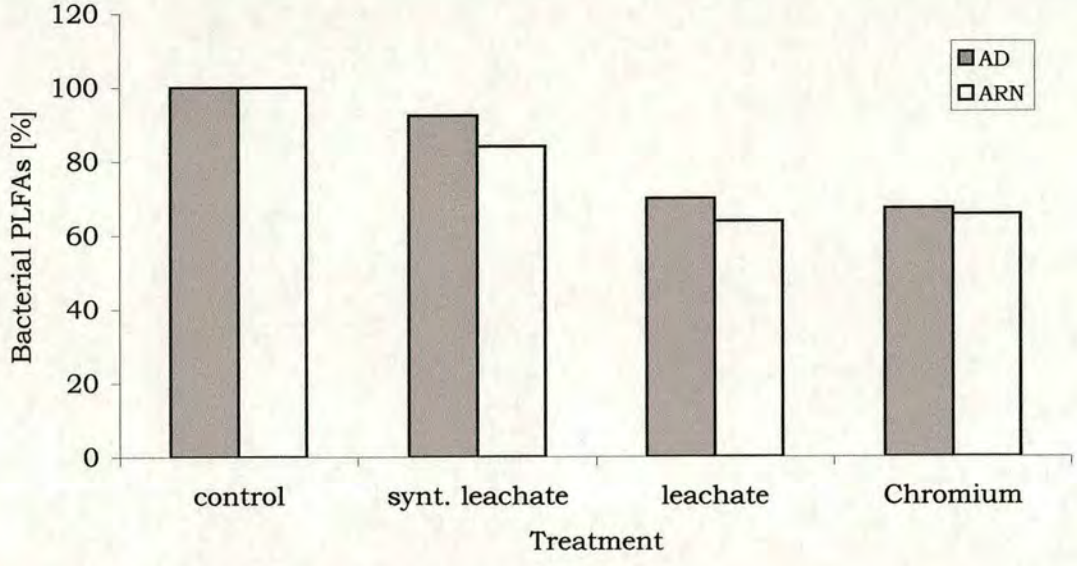


Fig. 6.16 Bacterial PLFA content (relative to control) of two soils treated with four leachates control (deionised water); synthetic leachate without Cr, COPR leachate and a synthetic Cr(VI) solution.

The actinomycetes PLFAs (16:0 (10 Me), 17:0 (10Me), and 18:0 (10Me) (Chapman et al., 2000) were reduced by 20-30% in treatments containing Cr(VI), (Figure 6.17).

The effect of treatment and soil type on the actinomycetes PLFAs (16:0 (10 Me), 17:0 (10Me), and 18:0 (10Me)) was highly significant (P= 0.001). The ANOVA separating soils showed that treatment had a slightly significant effect on the actinomycetes PLFAs in Aldroughty (P=0.173) and a highly significant effect on the actinomycetes PLFAs in Arnhall (P<0.001).

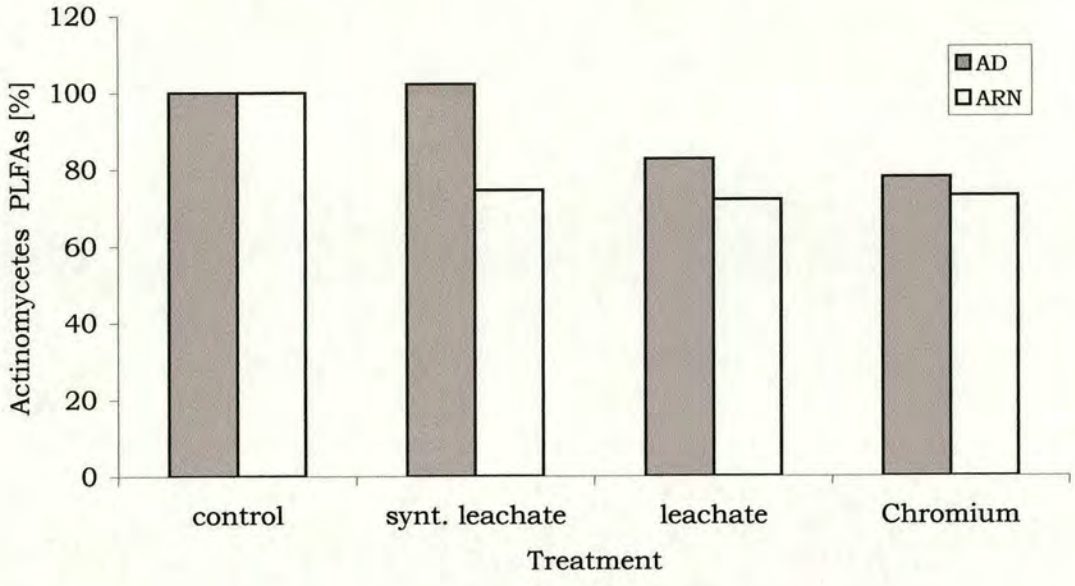


Fig. 6.17 Actinomycetes PLFA content (relative to control) of two soils under four different treatments: control (deionised water); synthetic leachate without Cr, COPR leachate and a synthetic CrVI solution.

Gram-positive bacterial PLFAs (16:0(10 Me), 17:0(10Me), 18:0(10Me), 15:0i, 15:0a, 16:0i, 17:0i and 17:0a) (Yao *et al.*, 2000), were similar to that of bacterial PLFAs, with a reduction of 30-35% when treatments containing Cr(VI) where used (Figure 6.18).

The ANOVA using individual soils as blocking factor, showed a significant effect of treatment on the Aldroughty Gram-positive PLFAs (16:0(10 Me), 17:0(10Me), 18:0(10Me), 15:0i, 15:0a, 16:0i, 17:0i and 17:0a) ($P=0.036$) and a highly significant effect of treatment on the Arnhall Gram-positive PLFAs ($P<0.001$).

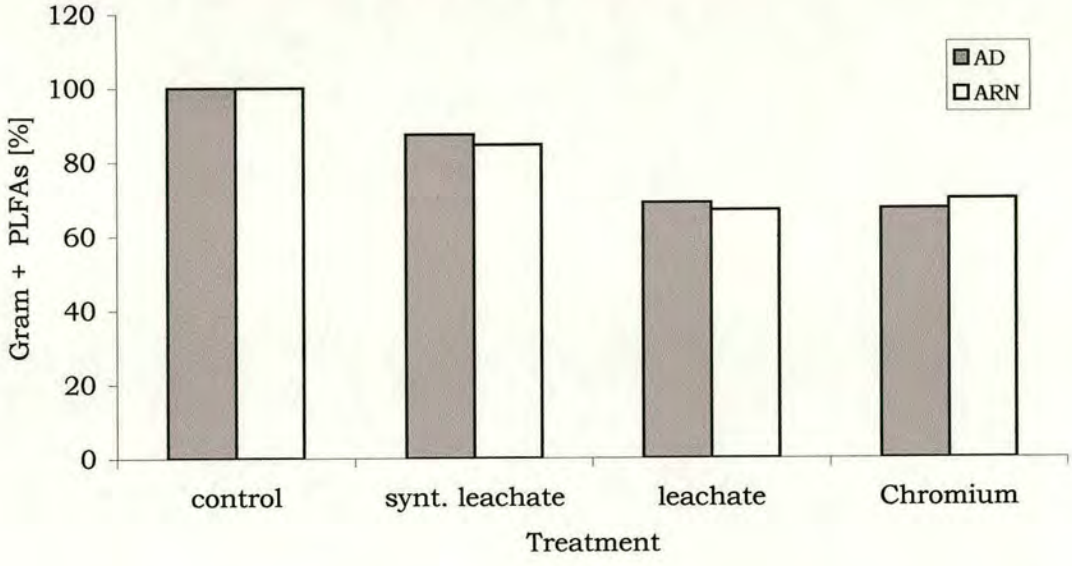


Fig. 6.18 Gram-positive PLFA content (relative to control) of two soils under four different treatments: control (deionised water); synthetic leachate without Cr, COPR leachate and a synthetic Cr(VI) solution.

The effect of treatments on the Gram-negative PLFAs (16:1ω5, 16:1ω7, 16:ω9, 17:0cy, 18:1ω5, 18:1ω7 and 19:0cy) is shown in Figure 6.19. A reduction between 30 and 37% was shown on the PLFAs from soils treated with Cr(VI). The treatment at high pH also caused a reduction on Gram-negative PLFAs between 10 and 20%.

The ANOVA showed a highly significant effect of treatment on the Gram-negative bacteria PLFAs (16:1ω5, 16:1ω7, 16:ω9, 17:0cy, 18:1ω5, 18:1ω7 and 19:0cy) in both Aldroughty ($P=0.005$) and Arnhall ($P<0.001$).

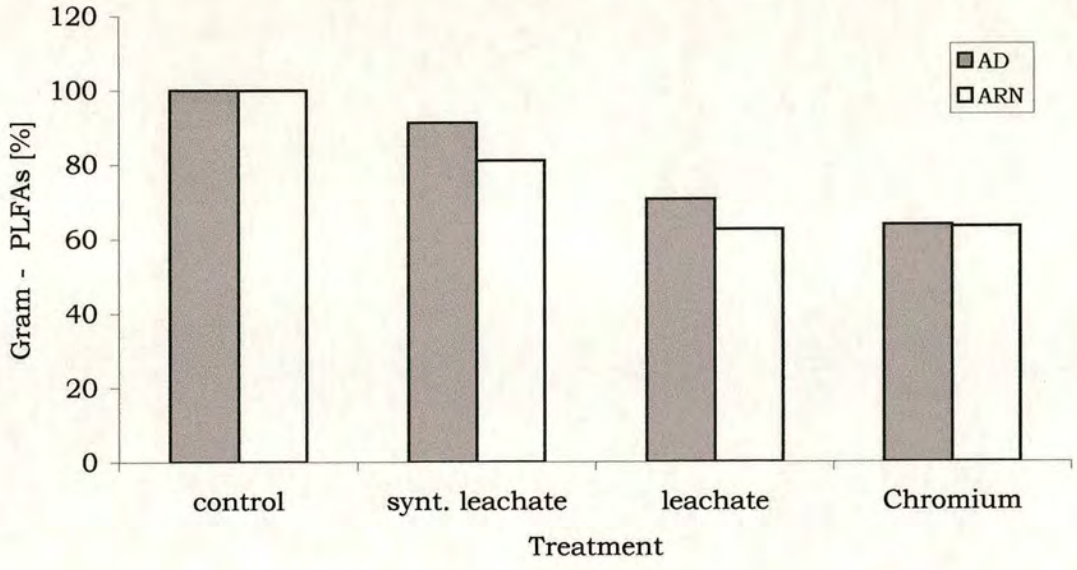


Fig. 6.19 Gram-positive PLFA content (relative to control) of two soils under four different treatments: control (deionised water); synthetic leachate without Cr, COPR leachate and a synthetic Cr(VI) solution.

The ratio between Gram-positive and Gram-negative bacteria was not changed significantly with treatments (data not shown).

The PLFA 18:2ω6,9 was used as fungal indicator (Frostegård and Bååth, 1996). and again a reduction in the relative amount was observed in treatments that contained Cr(VI), with the highest reduction caused by the Cr(VI) synthetic solution treatment (Figure 6.20). The reduction levels were similar to those observed in bacterial PLFAs, and the effects of Cr(VI) treatment in both soils were similar.

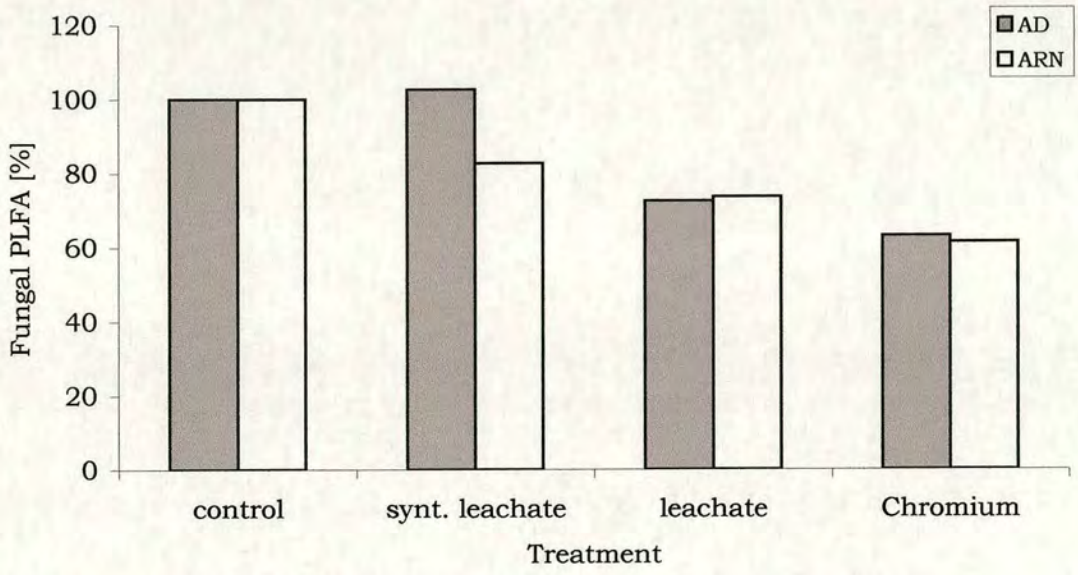


Fig. 6.20 Fungal PLFA content (relative to control) of two soils under four different treatments: control (deionised water); synthetic leachate without Cr, COPR leachate and a synthetic Cr(VI) solution.

A highly significant effect ($P<0.001$) of treatment was also found on individual PLFAs, including: 14:1w9c, 16:0br, 16:0i, C16:1w11t, C16:1w7c, 16:1w5, 17:0br, 17:0cy, 17:1w8t, 18:0br, 17:0(10Me), 18:1w9, 18:1w10 or 11, 18:0(10Me),19:1w8, 19:0, 20:4 (5,8,11,14), 20:5w3, 20:4 (6,10,14,18), 20:1w9 and 20:0.

The amount of PLFAs 16:0br, 16:0i, 17:0br, 17:0cy, 18:0br, 17:0(10Me), 18:0(10Me), 19:1w8, 19:0 and 20:0 increased in soils which had been treated with Cr(VI).

The effect of soil pH on PLFAs was studied separately comparing it with the control (without Cr(VI) treatments). It was found that the effect was significant on the PLFA groups, total PLFA ($P=0.033$), bacterial PLFA ($P=0.041$), actinomycetes PLFA ($P=0.032$), Gram-

positive (P=0.044), Gram-negative (P=0.015). This indicates again, the difficulty in separating the toxic effects of Cr(VI) from those of soil pH.

6.5.5 Effects on the metabolic capacity of microorganisms

If the metabolic capacity of soil microorganisms is impaired (Section 5.6.3), soil functioning will potentially be reduced. In Chapter 5 the study of the community structure was complemented with the study of the metabolic capacity of the microbial communities. The metabolic capacity could potentially give an indication of the groups of organisms that grow better or are more sensitive to contaminants. This section reports on the analysis of the metabolic capacities of microorganisms in the same samples of Arnhall and Aldroughty from the experiment described earlier in this chapter. In this section the effects of COPR leachate on the metabolic capacity of microorganisms were analysed.

Carbon sources used in the test plates included the sugars (L-arabinose, fructose, D-galactose and D-glucose), amino acids (L-alanine, aspartic acid, cysteine, L-lysine and serine) and carboxylic acids (citric acid, γ-amino butyric acid, malic acid, N-acetyl glucosamine, oxalic acid and 3,4-dihydroxybenzoic acid). A blank control with water-only added was also included. Each sample was tested in triplicate.

The respiration (production of carbon dioxide in the control) and substrate induced respiration (SIR) was measured for each carbon source in the different soils. The overall production of carbon dioxide was calculated from the sum of individual carbon sources for each treatment as a measure of overall activity. In general, it was observed that microorganisms in the controls of Arnhall produced a

higher amount of CO_2 than microorganisms in the controls of Aldroughty. The pH treatment (synthetic leachate) produced a similar respiration to that of the control, while treatments with Cr decreased respiration. In Aldroughty the COPR leachate had the greatest effect, reducing the respiration by 50 % (compared with the controls), while in Arnhall the Cr(VI) synthetic solution had the highest effect, reducing the production of CO_2 to 55 % of that of the control (to 70 % in the case of the COPR leachate).

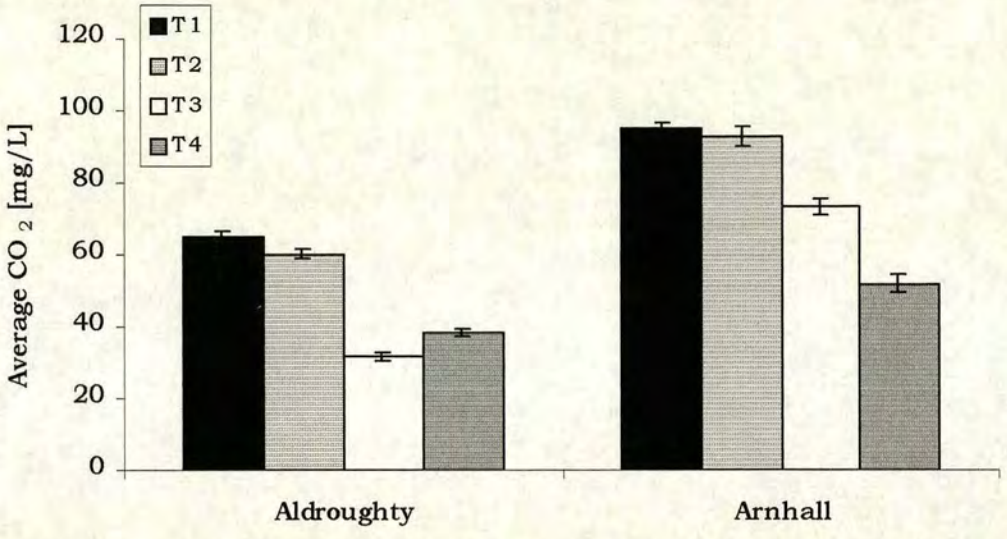


Fig. 6.21 Substrate induced respiration of microorganisms in two soils under four different treatments. T1= control, T2= Synthetic Leachate, T3= COPR leachate, T4= Cr(VI) synthetic solution.

The SIR data was analysed by principal components. The plot of PC2 versus PC1 (Figure 6.22) showed that there was a clear difference in the respiration of the soils and that the Cr treatments could be separated from the non-Cr treatments. In Aldroughty it was also possible to distinguish clearly between the COPR leachate and the Cr(VI) synthetic solution, although in Arnhall this difference is not as obvious.

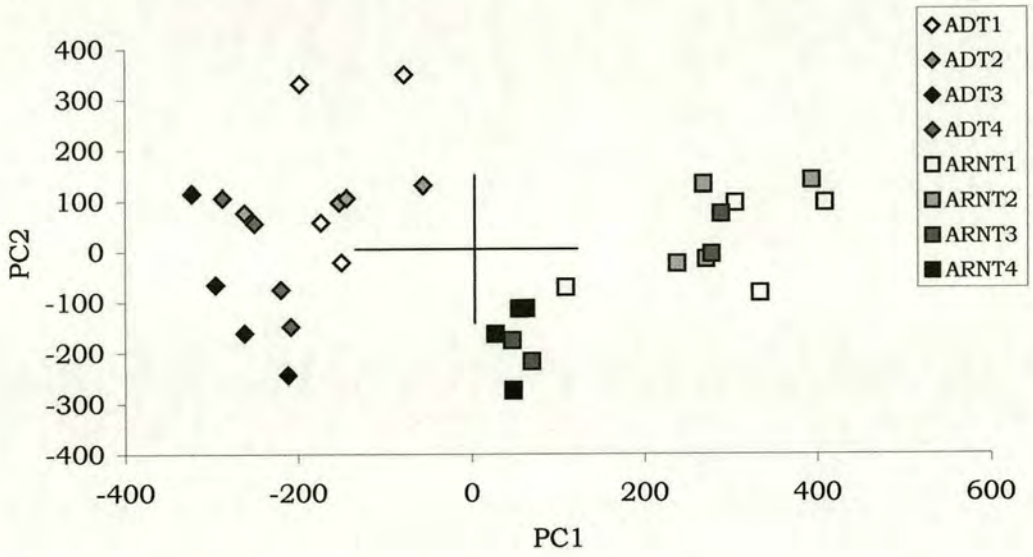


Fig. 6.22 Plot of PC1 scores vs PC2 scores from the PCA analysis of substrate induced respiration data for Aldroughty and Arnhall. Data is separated by treatment and soil, where AD= Aldroughty; ARN= Arnhall; T1= Control; T2=Synthetic Leachate; T3= COPR leachate and T4= Cr(VI) sysnthetic solution.

The utilisation of sugars by microorganisms in Aldroughty (Figure 6.23a) was lower than that of microorganisms in Arnhall (Figure 6.23b), but in both cases the Cr(VI) treatments (COPR leachate and Cr(VI) synthetic solution resulted in a lower production of CO_2. The reduction in CO_2 due to COPR leachate was greater in the Aldroughty soil, with fructose, galactose and glucose showing a reduction as high as 80-85 % (compared to the control). The reduction in overall SIR by the microorganisms in Arnhall was between 30 and 60 %.

The utilisation of amino acids was different. Microorganisms in the controls utilised cysteine and lysine in a similar way, and a higher utilisation of alanine, aspartic acid and serine were recorded in Arnhall compared with Aldroughty..

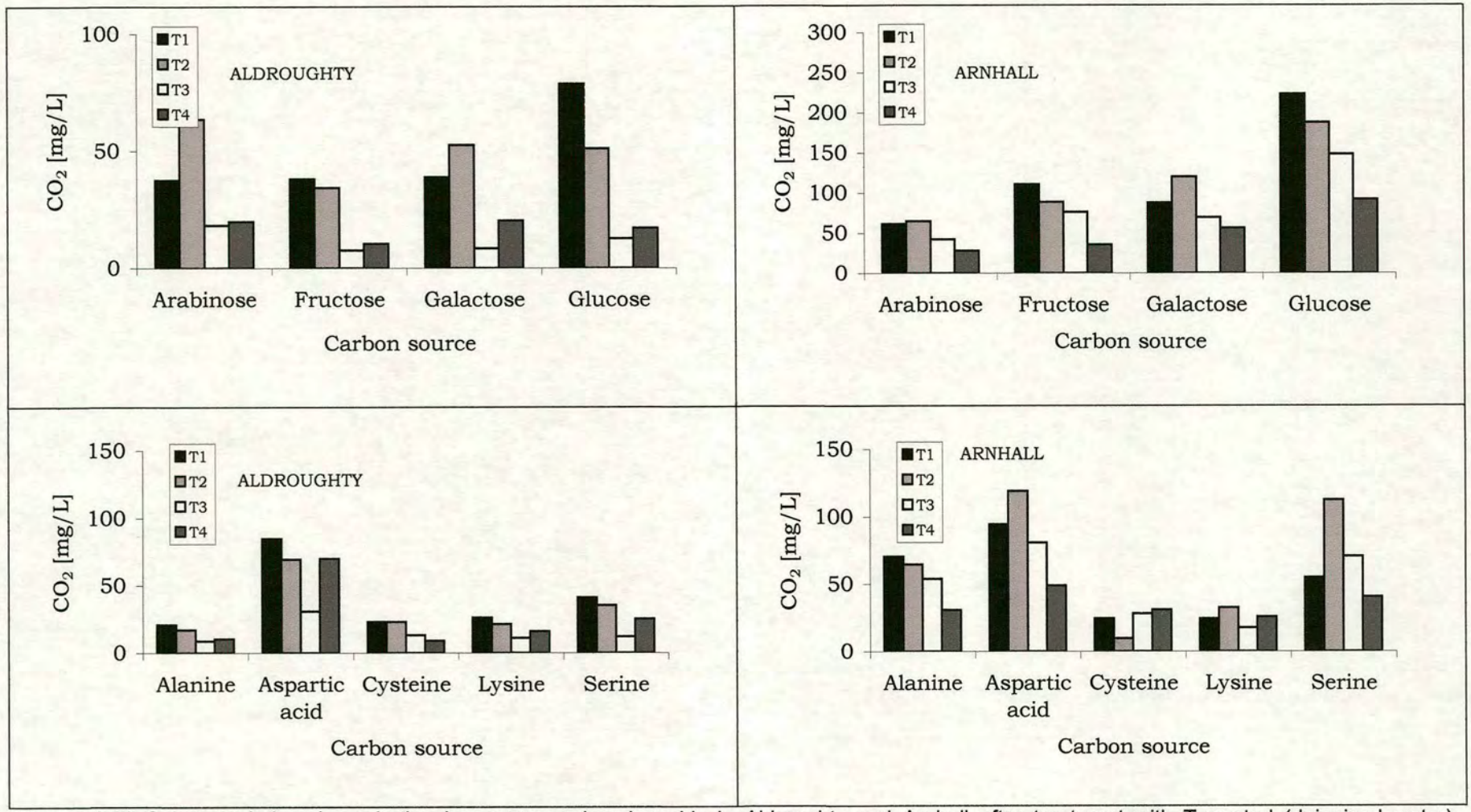


Figure 6.23 Substrate induced respiration by sugars and aminoacids in Aldroughty and Arnhall after treatment with T=control (deionised water), T=Synthetic Leachate; T3= COPR leachate and T4= Cr(VI) synthetic solution.

The chromium treatments (COPR leachate and Cr(VI) synthetic solution) decreased SIR from the amino acids in Aldroughty (Figure 6.23c) and Arnhall (Figure 6.23d), with the exception of cysteine and serine in Arnhall, for which the Cr treatments increased SIR above the level of the controls. The reduction in SIR was again greater than in Aldroughty (up to 89 % for lysine).

The utilisation of oxalic and malic acid in Aldroughty resulted in a high production of CO_2 (Figure 6.24). Again, the reduction of respiration was obvious in soils treated with Cr(VI). In general, the COPR leachate caused the highest reduction of respiration in Aldroughty and the Cr(VI) synthetic solution the highest reduction of respiration in Arnhall (Figure 6.25).

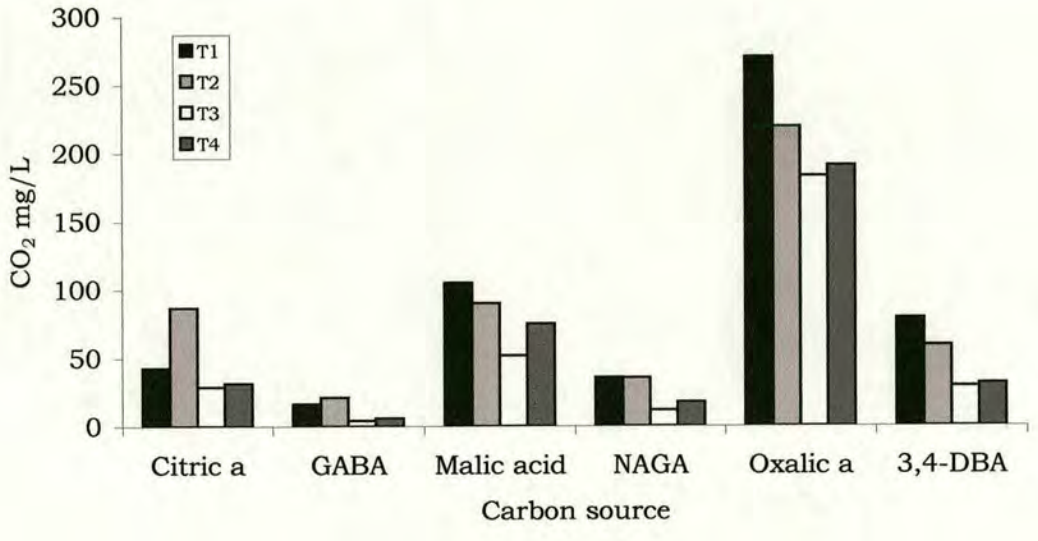


Fig. 6.24 Substrate induced respiration resulting from carboxylic acids in Aldroughty soils treated with T1=control, T2=Synthetic Leachate, T3=COPR Leachate and T4=Cr(VI) synthetic solution. GABA= γ-amino butyric acid; NAGA= N-acetyl-glucosamine; 3,4-DBA=3,4-Dihydroxybenzoic acid.

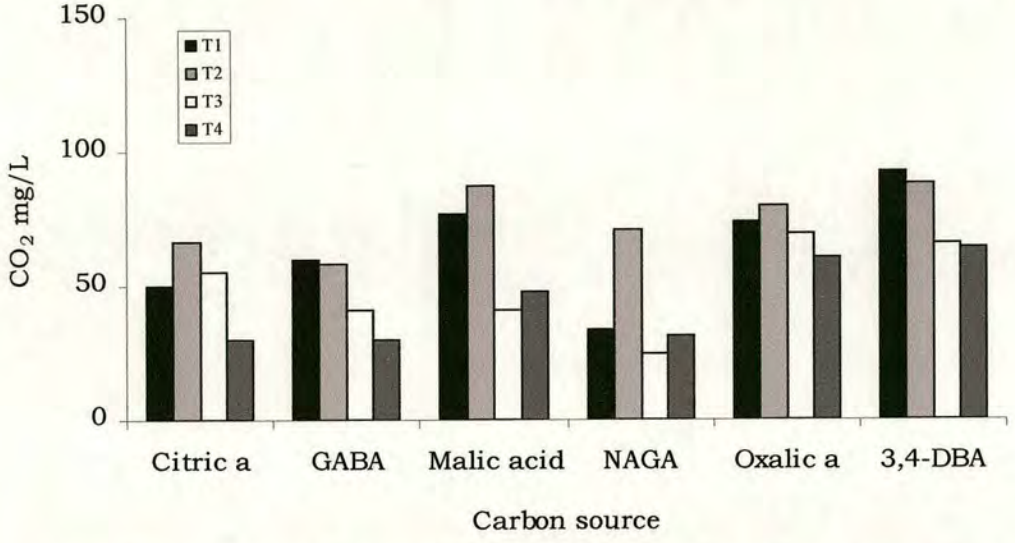


Fig. 6.25 Substrate induced respiration resulting from carboxylic acids in Arnhall soils treated with T1 = control, T2 = synthetic leachate, T3 = COPR leachate and T4 = Cr(VI) synthetic solution. GABA= γ-amino butyric acid; NAGA= N-acetyl-glucosamine; 3,4-DBA=3,4-Dihydroxybenzoic acid.

The reduction on respiration in Aldroughty was again more obvious, with γ-amino butyric acid producing the lowest respiration (reduction of CO_2 of 74 % compared with the control).

In general it was observed that the utilisation of carbon sources (with the exception of oxalic and malic acid) was lower in the controls of Aldroughty than in the controls of Arnhall. The highest production of CO_2 in Aldroughty was observed for the utilisation of carboxylic acids while the highest production of CO_2 in Arnhall resulted from the utilisation of sugars, in particular glucose.

It was also observed that in general, Cr(VI) treatments caused a greater reduction of CO2 in Aldroughty than in Arnhall. The COPR leachate was responsible for the greatest reduction in Aldroughty

soil. The synthetic Cr(VI) treatment produced the greatest reduction of CO_2 in Arnhall.

6.5.6 Toxicity of phosphate extracts to E. coli pUCD607

The toxicity of phosphate extracts obtained from the different soils was tested with *E. coli* pUCD607 and compared with the respective control soil (non Cr(VI) added) (data not shown). The results showed that there was not a significant difference between treatments (P=0.800) among the four treatments. Phosphate extracts contained very low concentrations of Cr(VI), to which the biosensor was not sensitive.

6.6 Discussion

The chemical composition and Cr(VI) adsorption capacities of the soils used in this experiment were reported in Chapter 4. Aldroughty had the smallest adsorption capacity and a pH over 7.0. The relatively high pH in Aldroughty might have caused a stronger retention of nutrients in the soil, which could explain why plants growing in Aldroughty started to turn yellow after a couple of weeks of growth (before any treatments were applied).

Nutrient deficiencies in crops growing in soils over pH 6.0 have been reported previously, *e.g.* Mn is the most commonly deficient element in field crops in England and Wales. Deficiency is common on sands and loamy soils, but less severe than in mineral soils, where the deficiency is associated with pH above 6.5. Other examples of

nutrient deficiencies in crops growing in soils over pH 6.5 include B, Fe and Zn (ADAS, 1983).

The nutrient solution applied to the soils could have had an effect on the toxicity of treatments to microbial communities and plants, but this was not investigated. Application of fertilisers is common under field conditions, therefore the use of the nutrient solution contributed to the simulation of field conditions.

The application of treatments (deionised water, synthetic leachate, COPR-leachate and Cr(VI) synthetic solution) resulted in a significant change in soil pH ($P<0.001$). In both soils the greatest changes were associated with the application of the synthetic leachate, followed by the soils treated with the COPR leachate (Figure 6.3). Surprisingly, the pH of the Cr(VI) synthetic solution (9.5) was much lower than the pH of the COPR-leachate (12.1). .

Apart from soil pH, the application of treatments had significant effects on the soil contents of Al ($P=0.098$), Cr ($P<0.001$), K ($P=0.013$), Mn ($P<0.001$), Na ($P<0.001$), Se ($P<0.001$) and Zn ($P=0.075$)

In both soils, the concentrations of soluble and exchangeable Cr(VI) found in phosphate extracts of soils treated with the COPR leachate were higher than those in phosphate extracts from soils treated with the Cr(VI) synthetic solution. The concentrations of Cr(VI) found in phosphate extracts were higher in Arnhall than in Aldroughty. This could, at first sight, contradict the findings on Cr(VI) adsorption capacity in Chapter 5. Nevertheless, the presence of plants could have resulted in changes in the chemical composition of soil solution and the rhizosphere zone (Khan et al., 2000).

The application of treatments also had effects in the weight and composition of roots and shoots. Roots growing in controls grew more successfully in the soils free of Cr(VI). The lowest biomass was recorded for COPR leachate treatment in both soils.

Effects of treatments on the chemical composition of leaf biomass were also recorded. For example the concentrations of Al, Fe, K, Mn and Zn in leaves from plants growing in Aldroughty and watered with the Cr(VI) treatments were lower than in the leaves from plants watered without Cr(VI). The leaves from plants watered with the Cr(VI) treatments and growing in Arnhall were lower in K, Mn, S and Zn than in the soil watered with solutions without Cr(VI).

Chromium was detected in the leaves of plants living in both soils, but Aldroughty showed a higher concentration of Cr (70 mg/kg) than Arnhall (33 mg/kg). Leaves of plants in control soils and synthetic leachates in both soils had levels of Cr close to zero.

Chromium concentrations in the roots were much higher than the amount translocated to leaves, with concentrations of around 1450 mg/kg in the roots from plants growing in Aldroughty and 750 mg/kg in the roots growing in Arnhall. The higher accumulation of Cr in the roots by Aldroughty could then explain why the concentrations of Cr(VI) in soil phosphate extracts were lower in Aldroughty than in Arnhall.

Roots apparently accumulated more Cr than leaves in both soils. The speciation and actual amount of Cr(VI) was not studied in the plant material, therefore it is not known if any of the Cr found by the ICP analysis had been converted to Cr(III). It would be interesting to investigate whether the plant system, possibly in the roots, convert Cr(VI) into Cr(III). The accumulation of Cr in the roots of plants in

this experiment agrees with other studies. Pulford *et al.* (2001) reported that the uptake of Cr in a range of tree species occurred mainly in the roots and was poorly transported into aerial tissue. Lytle *et al.* (1998), using X-ray spectroscopy, found that *Eichhornia crassipes* (water hyacinth), supplied with Cr(VI), accumulated non-toxic Cr(III) in root and shoot tissues. In general, it has been suggested that roots accumulate 10 to 100 times more Cr than shoots and other tissues (Cervantes *et al.*, 2001). High values of accumulation in roots (160-350 mg/kg) have also been reported in plants that can uptake high amounts of sulphur (Zayed *et al.*, 1998). Values found in this work can be considered high, compared with the accumulation of Cr in *Betula* (Birch) and *Humulus lupulus* (Hop) leaves of around 13 mg/kg reported by Stępniewska and Bucior (2001), although accumulations of up to 6000 mg/kg have been observed in the roots of wetland plants such as *Eichhornia crassipes* (Lytle *et al.*, 1998).

Visual symptoms of plants also gave an indication of stress and possibly nutrient deficiencies (ADAS, 1983), which indicated that even at concentrations as low as 50 mg/kg, plants which are already established can be affected, if in contact with Cr(VI)

In general, it was observed that Cr(VI) treatment affected the microbial community since lower amounts of PLFAs were found in the Cr(VI) contaminated soils than in the non-contaminated soils. However, it was not clear which treatment affected the microorganisms the most. The COPR leachate treatment seemed to cause the biggest reduction in PLFAs for microorganisms present in Arnhall, while the synthetic Cr(VI) treatment caused the highest reduction of microbial groups in Aldroughty.

The effect of Cr(VI) treatments on total and bacterial PLFAs (including Gram-positive and Gram-negative), was very similar. The effect of Cr(VI) treatments on actinomycetes seemed less severe than in bacteria.

The amounts of PLFAs 16:0br, 16:0i, 17:0br, 17:0cy, 18:0br, 17:0(10Me), 18:0(10Me), 19:1w8, 19:0 and 20:0 increased in soils which had been treated with Cr(VI), probably indicating that the microorganisms containing these PLFAs were part of a population resistant to Cr(VI). A variety of chromate-resistant isolates has been reported, *e.g. Pseudomonas fluorescens* (Ohtake *et al.*, 1987) and *Alcaligenes eutrophus* (Nies *et al.*, 1989).

Again, Cr(VI) seemed more toxic when added to Arnhall than when added to Aldroughty. In Chapter 4, toxicity curves were obtained for the different groups of PLFAs in Arnhall (using Cr(VI) added as explanatory variable) The comparison of expected and experimental values of PLFAs remaining in the soil when 50 mg/kg of Cr(VI) were added, showed that these samples were slightly more toxic than expected from the more controlled experiment (Table 6.4). The contribution of different components in the leachate, the presence of plants and the different metabolic activities of bacteria under these conditions could have affected the toxicity of the sample

Table 6.4 Comparison of actual and calculated PLFAs when adding 50 mg/kg Cr(VI) to Arnhall soil (according to calculated values from toxicity curves obtained from the microcosm experiment).

PLFA group	Actual remaining percentage PLFA in the soils (from pot experiment)	Expected remaining percentage PLFA (calculated from microcosm toxicity curves)
Total	65	74
Bacteria	65	69
Actinomycetes	72	83
Gram-positive	68	74
Gram-negative	62	68

The production of CO_2 was in general higher in Arnhall than in Aldroughty soils. The differences in substrate induced respiration (SIR) in the controls of both soils (*i.e.* Cr(VI) added) showed that these soils had microbial communities with different metabolic capacities. This is consistent with the PLFA analysis of the soils which showed a difference in community structure between soils. For both soil types, the metabolic capacity of microorganisms was significantly affected by Cr(VI) added at concentrations of approximately 50 mg/kg. However, the effects of COPR leachate were not easily distinguished from the effects of the synthetic Cr(VI) solution.

The community physiological profiles showed that Cr(VI) was more toxic when added to the soil with the lowest Cr(VI) adsorption than when added to the soil with the highest adsorption capacity (opposite to what was observed with the PLFA analysis). The respiration induced by most of the carbon sources in Aldroughty was reduced by more than 50% compared with the control. Suggesting that perhaps Cr(VI) did not change drastically the microbial community structure in Aldroughty, but it could have impaired the functionality of some of the species present, while Cr(VI) in Arnhall soil might have had more changes in the community structure. The changes in microbial community functionality in Aldroughty soil might also be related to the high concentrations of Cr found in the roots, perhaps detoxification mechanisms would produce modified conditions which might impair the normal functions of the community.

The utilisation of cysteine was not particularly high as happened in the microcosm experiment, again probably the presence of plants

provided a different environment, which resulted in the ability of different species to grow.

There was not a clear effect of treatment pH in the respiration observed, suggesting that toxic effects were related mainly to Cr(VI).

The results from the bioassays using *E. coli* pUCD607 showed that the biosensor was not sensitive to the low concentrations of Cr(VI) in present in the phosphate extracts, while plants and microbial communities were sensitive to the Cr(VI) added to the soil. This may have been related to the method used to characterise available Cr(VI), and it highlights the fact that *ex situ* bioassays, which depend on extraction methods might not describe the *in situ* effects of Cr(VI) in contaminated sites.

It was found that plants and microorganisms were affected by the addition of 50 mg Cr(VI)/kg soil. Currently in the UK agricultural soil quality is protected by allowing a maximum of 400 mg/kg of total Cr to be added to soils (Scott, 2002). If Cr(VI) species were around 10% of the total Cr, the current limits would be very close to the concentrations used in this experiment, with which toxic effects have been observed. Therefore, the review of current limits may be necessary to protect microbial communities and preserve the balance of microbial mediated soil processes like carbon and nitrogen mineralisation (Nordgren *et al.*, 1986; Chander *et al.* 1995).

6.7 Conclusions

The sections presenting the results for soil physicochemical analyses, microbial analyses and plant analyses, showed that in

general, Cr(VI) at the concentrations added to the soils (50 mg/kg) had a toxic effect at all levels, impairing the metabolic capacity of microorganisms, their relative abundance, community structure and the development and elemental composition of plants and soil physicochemical properties. Consequently this level of contamination had very significant effects on the soil-plant system.

Based on the hypotheses formulated at the beginning of the experiment, the following conclusions can be drawn:

- COPR leachate did affect soil physicochemical properties (pH, elements in soil solution).

- COPR leachate did have a toxic effect on established "healthy" barley plants at the tillering/stem development stage.

- COPR leachate did have a toxic effect on soil microbial populations, both in the relative abundance of microorganisms and their metabolic capacity.

- The toxicity was detected at low levels of Cr(VI) addition, approximately 50 mg/kg soil.

- Cr(VI) seemed to be the major factor contributing to toxicity, The PLFA analysis showed Cr(VI) was more toxic when added to Arnhall than when added to Aldroughty; the opposite was observed from the CCP analysis.

- The effect of COPR leachate and synthetic Cr(VI) solution seemed to be very similar and often not distinguishable,

supporting the hypothesis that Cr(VI) was the major factor contributing to toxicity.

- The toxicity of Cr(VI) to microorganisms was slightly higher in this experiment compared with observations from the microcosm experiment. Greater toxicity to microorganisms was recorded in the system with plants.

- Some microorganisms in the community (as represented by their PLFAs) increased with Cr(VI) addition and may represent species resistant to Cr(VI)

- It was also found that Cr(VI) accumulated in the roots of plants in higher amounts than in the leaves and that higher levels of Cr were accumulated in barley roots. It was not investigated if the Cr that had been sorbed by the roots and that its presence in the leaves had been altered from Cr(VI) to Cr(III), a possible detoxification mechanism. The zone of soil immediately surrounding the roots, called the rhizosphere, can have lower pH and higher anaerobicity (Griffiths et al., 1994), which may also have altered the speciation of Cr.

- Similarly, the plants did not seem to be affected by the slight change of soil pH, and the effect on microorganisms was not very obvious. This might also indicate that chronic exposure of Cr(VI) has a more important effect on the organisms than pH.

Chapter 7

General Discussion

The experiments carried out as part of this work were designed to study the (eco)toxicity of chromium in soils using a combination of chemical and biological analyses. Chemical analyses cannot provide by themselves ecotoxicological information, and microorganisms, despite having proved to be good indicators of the toxicity of aquatic and terrestrial systems, usually cannot describe their composition. In addition, if toxicity is considered as a complex continuum of biochemical, physiological, whole organism, population and community responses among a broad diversity of living organisms (Luoma, 1995), the use of a combined chemical and biological approach has the advantage of taking into account the different factors that may contribute to the (eco)toxicity of a contaminant.

Chromium, although discovered only relatively recently has found many industrial applications, which in turn have led to important localised contamination; between 500 and $1300x10^6$ kg of chromium are released into soils every year (Nriagu and Pacyna, 1988).

Most concern over chromium contamination is associated with Cr(VI), which can cause respiratory cancer (IARC, 1990) but it has also been found toxic to some bacteria, algae [Wong and Trevors, 1988) and plants (Cervantes *et al.,* 2001). On the other hand, it is recognised that the other important Cr species in the environment, Cr(III), is essential for glucose metabolism in mammals (Friberg *et al.*, 1986). In addition there is recognition that risk assessment must also consider the wider environmental effects of pollutants on the general ecology of receiving ecosystems but the emphasis for Cr has always been directed to human health risks. The risks to different

organisms depend on the bioavailability of Cr and its relative toxicity to them.

Chromium speciation determines the mobility of chromium in the environment and the likelihood of causing damage to ecosystems. In addition, Cr species can interconvert both in the environment and under laboratory conditions. Therefore, the study of Cr (eco)toxicity by the use of chemical analysis alone cannot be representative of what happens in the field; in this sense, biological analysis in combination with chemical analysis can give a better indication of the bioavailability and (eco)toxicity of this potentially toxic element in environmental samples.

The chemical manipulations and analyses used in this work had the purpose of characterising environmental samples including soils, urban soils and ground water. Routine chemical manipulations and analyses included the measurement of pH; digestions of the environmental samples; extractions of exchangeable Cr(VI) (phosphate extractions) and total Cr(VI) in soils (by alkaline digestions); extraction of soil pore waters (by centrifugation); and the use of ICP-OES and colorimetric methods to determine the concentrations of elements and Cr(VI) in solution, respectively.

As previously mentioned, bioassays study the response of an organism(s) to a contaminant(s) and can involve many different biological mechanisms, both natural and genetically induced. Response can be assessed at any level of biological organisation through the use of single species, a battery of single species or communities exposed to the same analyte (Luoma, 1995). In this work both single species bioassays and community bioassays were used to determine acute and chronic toxicity and *ex situ* and *in situ* toxicity of Cr(VI).

The first stage of the work (Chapter 3) used a single species biosensor, a bioluminescent construct (pUCD607) of *Escherichia coli* (*E. coli*), to investigate the toxicity of Cr(VI) in solutions prepared in the laboratory at different pHs (by using a range of different Cr(VI) concentrations and constructing concentration-response curves). This biosensor was a simple and fast assay that tested the toxicity of samples in solution phase.

The concentration-response curves obtained at different pHs were used to calculate Median Effect Concentrations values (EC_{50}) at each pH. The comparison of the EC_{50} values obtained at pH 4.5, 5.8, 7.0, 8.0 and 9.0 (34, 41, 218, 417 and 311 mg/L Cr(VI), respectively) showed a decrease of toxicity with increasing pH up to a value of 8.0 (Chapter 3, Figure 3.11).

Comparison of the calculated EC_{50} values (Figure 3.13) and the reciprocal of EC_{50} values (Figure 3.14) with the speciation diagram of Cr(VI) suggested that the toxicity of Cr(VI) *to E. coli* pUCD607 was correlated with the distribution of $HCrO_4^{2-}$ (hydrogen chromate) ($r=0.85$, $P<0.001$): the higher the concentration of $HCrO_4^{2-}$ the higher the toxicity. Variations in the toxicity of chromium related to Cr(VI) speciation have been previously observed (Villaescusa *et al.,* 1997).

The observation that hydrogen chromate seemed to be the most toxic Cr(VI) species in solution could be the result of being a stronger oxidising agent than chromate (Kimbrough, 1999). Speciation, however, is also strongly dependent on pH, which can itself play an important role when assessing toxicity, as living organisms have a limited pH range where their metabolic functions can be carried out properly.

It was observed that there were differences between the response of the biosensor used in this work and the marine bacteria used by others (Villaescusa *et al.*, 1997) and that Cr was less toxic to luminescent bacteria than other potentially toxic elements such as Zn and Cd (Paton *et al.*, 1997; Campbell *et al.*, 2000). In some cases (Paton *et al.*, 1997) this was probably due to differences in the microbial species tested, but Campbell *et al.* (2000) also used *E. coli* pUCD607 and found that Cu and Zn were more toxic than Cr(VI).

The experiments in Chapter 3 suggested that the biosensor *E. coli* pUCD607 could be used to compare the toxicity of Cr-contaminated environmental samples but that careful comparisons were needed with pH reference solutions.

In Chapter 4 studies carried out on field samples were described. Farmer *et al.* (1999) have reported the contamination of some areas of South East Glasgow with chromite ore processing residue (COPR). The problem with these contaminated sites is normally that they are easily accessible by the local population; therefore there is a need to assess their potential risks and to search for remediation options, which in turn requires greater understanding of the chemical and biological process *in situ*.

Sixteen soil samples[1] were taken from a COPR-contaminated site and characterised. They varied in chemical composition, with major elements being Ca, Mg, Fe and Cr. Some samples contained up to 3% (w/w) total Cr and 0.4% (w/w) Cr(VI), greatly in excess of the trigger concentrations of total Cr and Cr(VI) allowed in the UK for park, allotments and open space of 1000 mg/kg and 25 mg/kg, respectively (ICRCL 59/83).

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[1] Urban soil samples

Samples contained different amounts of exchangeable Cr(VI), as determined by extraction with phosphate buffer solutions. Differences between these extractions and soil pore waters highlighted the need to define the bioavailable fraction of a contaminant (operational speciation), and demonstrated that the use of soil pore water might underestimate the toxicity of samples.

The Cr(VI) content of the soil phosphate extracts was found to be positively correlated with the amount of Ca, Mg and Fe in soil samples. Thomas *et al* (2001) have shown that Cr(VI) may well be present as an exchangeable anion in the hydrocalumite phase ($Ca_2(Al,Fe)(OH)_6(CrO_4)_{0.5}$) of COPR.

The toxicity of soil samples was tested through assays of phosphate extracts with *E. coli* pUCD607. Soil pore waters could not be used in this case, as some of them had prohibitively high pHs. The luminescence for different samples was compared with a control at pH 7.0 and with a control at an equivalent reference pH. The results showed that for concentrations above 4 mg/L Cr(VI) and pH above 8.3, the toxic effect was more obvious for phosphate extracts compared with reference pH controls than those compared to the pH 7.0 control, with toxicity increasing with increasing pH and Cr(VI).

It was difficult to separate the effect of pH from the effect of chemical composition on the toxicity of the phosphate extracts to *E. coli* pUCD607. Significant correlations ($P=0.01$) were found between phosphate extract toxicity (as compared with a pH 7.0 control) and the concentrations of Ca, Fe, Cr and Mg in soils, possibly again suggesting the release and role of exchangeable Cr(VI) in sample toxicity.

The results obtained from the bioassays on the phosphate extracts in Chapter 4 showed that the environmental samples were more toxic than the synthetic solutions containing the same amount of Cr(VI) (Chapter 3). EC_{25} values obtained in the experiments with synthetic Cr(VI) solutions in Chapter 3 showed that at pH between 8 and 9, a concentration of around 95 mg/L of Cr(VI) would be required to cause a 25% reduction in luminescence. The highest concentration of Cr(VI) found in phosphate extracts was 11 mg/L, but the toxicity recorded was higher than that of synthetic solutions at the same pH, as concentrations as low as 2 mg/L of Cr(VI) in phosphate extracts resulted in more than 25% reduction in luminescence.

The same difference in toxicity between synthetic Cr(VI) solutions and environmental samples was found when ground water samples collected from different COPR-contaminated sites were assayed with *E. coli* pUCD607 (Section 4.8.3). While EC_{50} values obtained for synthetic Cr(VI) solutions at pH 8.0 and 9.0 were 417 and 311 mg/L, respectively, there was a greater than 50% reduction in luminescence for some groundwater samples, none of which exceeded 30 mg/L Cr(VI).

The difference in the toxicities of synthetic solutions, compared with those of environmental samples, indicated that a combination of factors, including contaminant concentration, pH, matrix chemical composition and the interactions between the contaminant(s) and various other chemical species present in the matrix, can influence the toxicity of environmental samples. It is therefore difficult to just extrapolate the results from synthetic solution concentration-response curves to environmental samples. Also, the effect of pH matrix can be one of the major factors controlling toxicity.

This is also a good example of how chemical analysis only could not give accurate information on the toxicity of environmental samples. If just the concentration of Cr (or even Cr(VI)) had been used to indicate the toxicity of soil samples, the latter would have been underestimated. The feasibility of using a single species bioassay (*E. coli* pUCD607) and chemical analyses for comparing the toxicity of environmental samples, taking into account factors contributing to toxicity, was therefore demonstrated in Chapter 4. Nevertheless, this approach can only give an overview of acute toxicity to a biological species that might not represent accurately those present in the soil microbial communities.

Studies of the effects of contaminants on humans and single species are the most common, but there is still a gap in information related to the ecotoxicology (study of the toxic effects of chemical and physical agents on living organisms, especially on populations and communities within defined ecosystems (Butler, 1978)) of contaminants. Special attention should be paid to the study of effects of contaminants on soil microbial communities; if soil community functioning is impaired, nutrient cycles in the whole ecosystem can be affected (Giller et al, 1998).

In Chapter 5 a microcosm experiment was used to study the effect of adding increasing amounts of Cr(VI) (under relatively controlled conditions) on the microbial communities of three different soils. Soils (Aldroughty, Arnhall, Glencorse) were selected to reflect either different Cr(VI) adsorption capacity or the same Cr(VI) adsorption capacity but with a potentially different microbial community structure due to different land use. Since experiments in Chapter 3 and Chapter 4 had shown that the toxicity of Cr(VI) to *E. coli* pUCD607 was influenced by solution pH, it was also anticipated that the toxicity of Cr(VI) in the soils used was going to be correlated with

pH and the availability of Cr(VI) in soil pore water, as determined by Cr(VI) adsorption capacity and soil pH.

The toxicity of Cr-contaminated soil to indigenous microbial communities was studied through Signature Lipid Biomarkers (PLFA analysis, Section 5.6.2) and community-level physiological profiles (CLPP) (metabolic capacities of soil communities, Section 5.6.3) and compared with the results of the toxicity of soil pore waters to *E. coli* pUCD607 (Section 5.6.1) and the inhibition of root growth in *Hordeum vulgare* (Section 5.6.4).

The results from all bioassays in Chapter 5 showed that the toxicity of Cr in soils with different physicochemical properties varied depending on the type of bioassay employed. The response of *E. coli* pUCD607 bioassay and the plant assay to Cr(VI) addition to soils was different from that of the indigenous soil microbial communities.

When soils were assayed using *E. coli* pUCD607 the toxicity of Cr(VI) in the different soils increased as soil Cr(VI) adsorption capacity decreased, *i.e.* the toxicity increased in the order Arnhall < Glencorse < Aldroughty. Chromium (VI) added and Cr(VI) in soil pore waters seemed to be the most important factors controlling toxicity. The toxicity of Cr(VI) added to *Hordeum vulgare* seedlings exhibited a similar effect, with the soil of lowest adsorption capacity (Aldroughty) being more toxic than soils of a higher adsorption capacity. Both bioassays had a relatively similar sensitivity to Cr(VI); for the plant assay EC_{25} values for Aldroughty and Arnhall were 24 and 98 mg/kg, respectively; for *E. coli* pUCD607 EC_{25} values for Aldroughty and Arnhall were 31 and 110 mg/kg, respectively. It would be interesting to study with other plants if the use of *E. coli* pUCD607

could indicate the acute toxicity of Cr(VI)-contaminated soils to emerging seedlings.

On the other hand, when analysing the community structure using PLFAs, the order of toxic response of the soils was different. The overall structure of the soil community did not change significantly with the addition of Cr(VI) to Aldroughty. According to microbial biomass (Total PLFAs), the toxicity of Cr(VI) contaminated soils seemed to follow the order Arnhall (EC_{25}=47 mg/L) > Glencorse (EC_{25}=88 mg/L) > Aldroughty (no EC available) and according to Table 5.11, the most sensitive microbial guild was Gram-negative bacteria (EC_{25}=29 mg/kg in Arnhall, EC_{25}=56 mg/kg in Glencorse, EC_{25} not available for Aldroughty) and the least sensitive was fungi (EC_{25} =348 mg/kg in Arnhall, EC_{25}= 213 mg/kg in Glencorse, EC_{25} not available for Aldroughty).

It was also found that Gram-negative bacteria were more abundant than Gram-positive bacteria in the three soils. Gram-negative bacteria have been reported to dominate in metal-contaminated soils compared with Gram-positive bacteria (Hiroki, 1992; Wenderoth and Weber, 1999), but there exist also reports where Gram-positive bacteria in metal polluted soils dominate (Roane and Kellog, 1996) The ratio of Gram-positive to Gram-negative changed slightly in Arnhall and Glencorse towards more Gram-positive bacteria with the addition of Cr(VI). An increase of Gram-positive bacteria with increase in heavy metal pollution has been reported previously (Pennanen, 2001).

The PLFA profiles showed that non-amended (*i.e.* no Cr(VI) added) Aldroughty soil had a very different microbial community structure and lower microbial biomass (Figures 5.25 and 5.29) compared with those of Arnhall and Glencorse. The CLPP measurements also

showed that Aldroughty had the lowest respiration rate of the non-amended soils (Figure 5.46).

The CLPP respiration of soils with different Cr(VI) concentrations was in line with the effects of Cr(VI) on community structure. Arnhall, in general, had the lowest catabolic capacity, followed by Glencorse (Figure 5.51). It is interesting to note that Cr-contaminated Aldroughty had a much higher catabolic capacity than the last two soils and that activity for the consumption of cysteine, an aminoacid containing thiol groups was particularly high. The utilisation of cysteine in the non-contaminated soil was not as high as in the Cr(VI)-contaminated Aldroughty soils, probably indicating a shift in the contaminated soils towards microorganisms that can utilise S (higher concentrations of S in Aldroughty were found in the soil pore waters related to Cr(VI) addition).

It has been reported in the literature that Cr(VI) can enhance the growth and sulphate-reducing activity of sulphate-reducing bacteria (Karnachuk, 1995). Sulphate reducing bacteria can conserve energy by the dissimilatory reduction of SO_4^{2-} and a spore-forming reducing bacteria has also been found to grow with Cr(VI) in addition to various sulphur compounds as electron acceptors (Tebo and Obraztsova, 1998). If sulphate reducing bacteria could grow in Aldroughty, they would probably be able to detoxify some of the Cr(VI) by reducing it to Cr(III) (which could precipitate as $Cr(OH)_3$) (Lloyd *et al.*, 2001).

The smaller fluctuations in pH between treatments in the Aldroughty soil and the possibility of an adapted microbial population (possibly the presence of sulphate-reducing bacteria in higher amounts than in the other two soils, could also explain why a different toxicity is recorded for Aldroughty in the acute (*E. coli* pUCD607 and *Hordeum*

vulgare assays) and chronic assays (microbial community analysis). This would certainly merit further investigation.

The microcosm experiment was, to a certain degree, similar to the experiments from Chapter 3, in that concentration-response curves were obtained for Cr(VI) in synthetic solutions. The microcosm experiment was still subject to more control than natural systems in the environment as not only Cr(VI) was added as synthetic solutions, but also plants were not present in the system. The next step, therefore, was to study the effects of the addition to soil of Cr(VI) from leachate obtained from environmental samples, in the presence of plants, and to compare the response of soil microbial communities, plants and the biosensor *E. coli* pUCD607.

The two soils that had shown the highest toxicity when contaminated with Cr, Aldroughty (in acute assays) and Arnhall (in chronic assays) were planted with *Hordeum vulgare* seedlings. Plants were left to establish and then separated into four groups for different treatments: water, leachate from COPR-contaminated soils, a synthetic leachate without Cr and a synthetic Cr(VI) solution. Plants were watered with the same volumes of treatment solutions for 20 days (the total concentration of Cr(VI) added was 50 mg/kg) and then harvested prior to chemical and biological analysis of the samples.

The effects of soil type were soon observed in the plant development, *i.e.* prior to the addition of treatments, with plants growing in Aldroughty, with an alkaline pH, starting to turn yellow at the leaf tips after two weeks. In consequence, a nutrient solution was added to both soils to compensate for any nutrient deficiency.

When plants were harvested (after 20 days of applying the treatments), analysis of the plant tissue showed that there was a marked reduction of plant biomass in treatments containing Cr(VI), but that the highest reduction was recorded for plants treated with COPR leachate. Also there was a difference in Cr concentrations in leaves and roots, due to a combined effect of treatment and soil type. Chromium was accumulated in high concentrations in the roots of Aldroughty (approx. 1500 mg/kg) and less in Arnhall (approx. 750 mg/kg).

Visual symptoms in plants also gave an indication of stress and possibly nutrient deficiencies. When plants were harvested, the conditions of the plants were different to those before the treatments were applied (Figure 6.14), *i.e.* plants which had been treated with Cr(VI) were smaller than the untreated controls. The state of plants treated with Cr(VI) had obviously deteriorated both in Aldroughty (Figure 6.15) and Arnhall (Figure 6.16) soils.

The effect of the treatments on the soil microbial community structure (studied by PLFAs) was compared using the proportion of PLFAs (as expressed by mol percentage of the total PLFAs for each control). A decrease of around 30-35% in the total PLFAs was observed for the treatments containing Cr(VI) and around 5-15% for the synthetic leachate which had a higher pH. The decrease in total PLFA was greater in the Aldroughty soil than in the Arnhall soil. There was a highly significant effect ($P<0.001$) of treatment and type of soil on the total PLFAs (microbial biomass).

The effects of Cr(VI) in synthetic solutions on the different microbial guilds in the microcosm experiment (Chapter 5) were compared with the effects of Cr(VI) from COPR leachate on the microbial communities (Chapter 6). Again, the concentration of Cr(VI) applied

to the soil-plant system seemed to be more toxic than the one expected from the microcosm experiment, such that the toxic effects on the plants were indirectly affecting the microorganisms.

For both soil types the metabolic capacity of microorganisms was significantly affected by Cr(VI) added at concentrations of approximately 50 mg/kg soil. However, the effects of COPR leachate were not easily distinguished from the effects of the synthetic Cr(VI) solution. The soil with the more alkaline pH (Aldroughty) exhibited a greater toxic response to the leachate, probably as a result of the additive effect of pH and Cr(VI) and the presence of plants. It was also noted that the utilisation of cysteine was not as high as in the contaminated soils from the microcosm experiment, probably as a result of more complex processes occurring in soils with plants.

Cr(VI) at the concentrations added to the soils (approximately 50 mg/kg soil) exerted a toxic effect at all trophic levels tested (apart from E. colipUCD607 which was not sensitive to the low concentrations present in phosphate extracts). It impaired the metabolic capacity of microorganisms, their relative abundance, community structure and the development (and elemental composition) of plants and the physicochemical properties of soils. Consequently this level of contamination had very significant effects on the soil-plant system.

The results obtained from all experiments in soils suggest that relatively low levels of Cr(VI) can potentially impair the structure and functionality of soil microbial communities and also be toxic for *ex situ* bioassays. In the UK agricultural soil quality is protected by allowing a maximum of 400 mg/kg of total Cr to be added to soils (Scott, 2002). If Cr(VI) species were around 10% of the total Cr, the current limits would be very close to the concentrations used in the

pot experiment, with which toxic effects were observed. Therefore, the review of current limits may be necessary.

In order to find safe threshold values for Cr(VI) and total Cr in soils, a more extended study would be needed; a wider range of soils should be tested with community bioassays and simple chemical analysis and other relevant receptors should be included, e.g. other types of plants and nematodes.

Chapter 8

Conclusions

The experiments performed in this research led to the following conclusions:

- As the single species bioassay *E. coli* pUCD607 proved sensitive to Cr(VI), it was possible to use it to investigate the effects of Cr(VI) in solution. In this respect it was shown that Cr(VI) was toxic to the bacteria and that toxicity varied with solution pH; EC_{50} values calculated from concentration-response curves increased with pH up to pH 8.0, *e.g.* EC_{50} values at pH 4.0, 5.8, 7.0, 8.0 and 9.0 were 34, 41, 218, 417 and 311 mg/L Cr(VI), respectively. It was found that higher concentrations of hydrogen chromate ($HCrO_4^-$) were correlated with higher toxicity, but because the abundance of this species in solution is dependent on pH, it was difficult to separate the effects of speciation and pH on the apparent toxicity to *E. coli* pUCD607.

- It was possible to use the single species bioassay *E. coli* pUCD607 and chemical analyses to investigate the toxicity of Cr-contaminated samples but, when the toxicity of environmental samples was compared with the toxicity of synthetic solutions, it was found that the former were more toxic than expected, *i.e.* Cr(VI) in the environmental samples resulted in a higher toxicity than the concentrations of Cr(VI) in the synthetic solutions. It was found that a more complex interaction of chemical matrix and pH were the main contributing factors to toxicity and that bioavailable Cr(VI)

might have been influenced by the presence of exchangeable Cr(VI) in the solid samples and that the use of soil pore waters in this case might have underestimated toxicity.

- A controlled microcosm experiment was used to study the effects of added Cr(VI) on soil microbial communities of three different soils. The use of signature lipid biomarkers and community level physiological profiles for studying the structure of soil microbial communities and their metabolic capacity, showed that the addition of Cr(VI) in synthetic solutions to healthy soils had toxic effects on these communities. In general, the microbial biomass of contaminated soils decreased with the addition of Cr(VI). The community structure and metabolic capacity of microorganisms in contaminated soils changed compared with that of healthy soils, but the change was not necessarily correlated with the Cr(VI) adsorption capacity of soils, as had been predicted . Instead, more complex processes, probably involving changes in the chemical composition of the matrix and shifts in microbial species abundance (probably towards more Cr resistant species) might have determined the chronic toxicity of Cr(VI) to soil microbial communities. In conclusion, chemical methods on their own cannot predict the potential biological effects of Cr contamination of soil.

- Acute, *ex situ* single species bacterial and plant bioassays performed on the soils from the microcosm experiment showed that the acute toxicity of Cr(VI) in soils, where no plants were growing, was different to the chronic toxicity observed for soil microbial communities. In this case the acute toxicity of Cr(VI) to *E.coli* pUCD607 and to a plant assay was dependent on the Cr(VI) adsorption capacity of soils; toxicity of Cr(VI) was

greater in soils with lower Cr(VI) adsorption capacity. It was also found that young plants could be highly sensitive to Cr(VI) and that relatively low concentrations of Cr(VI) can impair the soil community structure in the long term. Consequently, a revision of the current regulatory limits may be appropriate.

- A more complex system (pot experiment) was used to study the effect of Cr(VI) on soil microbial communities where plants were growing. The addition of Cr(VI) in leachate from COPR-contaminated soils and Cr(VI) in synthetic solutions showed that Cr(VI) was toxic to soil microbial communities and plants at even the low levels used in this experiment (50 mg Cr(VI)/kg soil). Again, the chronic effects of Cr(VI) did not seem to be correlated with the Cr(VI) adsorption capacity of soils. The community physiological profiles indicated different results, Cr(VI) seemed more toxic in the soil with higher Cr(VI) adsorption capacity, suggesting the influence of a more complicated soil-plant-microbial interaction even though low concentrations phosphate extractible Cr(VI) in Cr-contaminated soils was less toxic than control soils. This highlighted again the dependence of *ex situ* bioassays on extraction methods.

- Overall, from the experiments carried out in this work, it can be concluded that the use of indigenous microbial communities (*in situ* bioassays) together with simple chemical analyses might be more indicative of local processes and account for more factors contributing to toxicity than chemical analysis alone or single species bioassays alone. The community bioassays used in this work are relatively simple and can be used to analyse samples in a relatively short time.

Therefore their use is highly recommended, especially to monitor remediation. The study can be complemented with the use of single species bioassays, bearing in mind that the observed toxicity will be greatly influenced by the extraction methods and that the resulting information will relate mainly to acute toxicity. Although this can be used for a quick comparison of toxicity of environmental sites, it will not reflect the more complex processes influencing toxicity on site.

Suggestions for further work

- To expand the study to a wider range of soils and receptors (by using other bioassays), in order to assess safe Cr(VI) threshold levels.

- To further investigate the possible shifts in microbial communities towards Cr resistant bacteria (e.g. sulphate reducing bacteria). There are specific methods for culturing and extracting sulphate reducing bacterial biomarkers that could be used to test this hypothesis.

- It would also be interesting to use both Cr(VI) and Cr(III) salts in long term studies to investigate potential effects of these two species in soil indigenous microbial communities.

- To test the approach at sites undergoing chemical, microbial and phytoremediation.

- To use the approach to study other type of chromium-contaminated sites, e.g. sites receiving tannery wastes which

could potentially have different pHs, and compare them with COPR-contaminated sites.

- To try the suggested approach first in another anionic species, e.g. to study the contamination of arsenic contaminated sites and later to expand it to other types of contaminant, e.g., persistent organic pollutants.

Appendix 1

Using the two-surface Langmuir equation to model Cr(VI) adsorption on soils

One approach to model chromate adsorption in soils is the use of the two-surface Langmuir equation (Zachara *et al.*, 1989), which has also been used for phosphate adsorption on soils (Holford and Wedderburn, 1978). Sposito (1982) concluded that for any experimental sorption isotherm $q(c)$, that is a smooth concave function of the concentration, c, that approaches a maximum value asymptotically, the Langmuir two-surface equation can be used to represent the sorption isotherm of an anion, regardless of the actual sorption mechanism. For these purposes an interpolation formula that relates the values of q(c) near the origin to its asymptotic behaviour is used. The same author also suggested that the chemical significance of this interpolation formula and therefore, of the two-surface Langmuir equation, cannot be evaluated in any way by the goodness of fit of the equation to experimental sorption data.

The two-surface Langmuir equation is described as:

$$q = \frac{b_1 K_1 c}{1 + K_1 c} + \frac{b_2 K_2 c}{1 + K_2 c} \qquad \text{equation 4.1}$$

Where b_1, K_1, b_2 *and* K_2 are adjustable parameters, which can be calculated using

$$y = \frac{\alpha_0 \beta_0 - \alpha_1 \beta_1}{\beta_0^{\,2} + \alpha_0 \beta_1} \qquad \text{equation 4.2}$$

and

$$x = \frac{\alpha_0^{\,2} + \alpha_1\beta_0}{\beta_0^{\,2} + \alpha_0\beta_1} \qquad \text{equation 4.3}$$

from Sposito (1982) derivations,

$$K_1 = \frac{1}{2}\left[y + (y^2 - 4x)^{1/2} \right] \qquad \text{equation 4.4}$$

and

$$K2 = \frac{1}{2}\left[y - \left(y^2 - 4x\right)^{1/2} \right] \qquad \text{equation 4.5}$$

solutions for b_1 and b_2 are provided by:

$$b_1 = \frac{\beta_0 K_1 + \beta_1 x}{K_1 - K_2} \qquad \text{equation 4.6}$$

and

$$b_2 = \frac{-\beta_0 K_2 - \beta_1 x}{K_1 - K_2} \qquad \text{equation 4.7}$$

The parameters α_0, α_1, β_0 and β_1, can be determined experimentally by plotting the sorption data in the form of a graph of q/c *versus* q. The ratio $K_d \equiv$ q/c is commonly termed the distribution coefficient for the sorption reaction.

Since the sorption isotherm is assumed to be concave towards the c-axis, K_d will be convex towards the *q*-axis. Therefore, a simple

inspection of the trend in a graph of K_d vs q will determine whether the interpolation theorem can be applied.

Figure A.1.1 illustrates the method for the determination of the parameters α_0, α_1, β_0 and β_1.

According to Sposito (1982), the slope of a graph of K_d against q approaches α_1/α_0 at low values of q and the intercept with the q-axis of a line through the data with this slope is equal to $\alpha_0^2/(-\alpha_1)$.
Therefore,

$S_1=\alpha_1/\alpha_0$
and
$I_1=\alpha_0^2/|\alpha_1|$

Where S and I refer to the slope and q-intercept of the initial, linear portion of a graph of K_d vs q, these equations can be solved for α_0 and α_1:
$\alpha_0=|S_1|I_1$
and
$\alpha_1=-S_1^2I_1$
α_0 also equals K_d as $q\downarrow 0$

K_d becomes asymptotically linear in q as $q\uparrow b$. This asymptotical linear portion has a slope $S_2=\beta_0/\beta_1$ and an intercept with the q-axis $I_2=\beta_0$, as illustrated in Fig A.1.1. Therefore,

$\beta_0=I_2$
and
$\beta_1 = I_2/S_2$

The extrapolation of a graph of experimental values K_d *versus* q to cut the q-axis results directly into the estimate of β_0, which is equal to b, the maximum value of $q(c)$, or q_{max}. This extrapolation method that makes use of only the experimental values of K_d, does not require the adherence of the sorption data to any particular isotherm equation.

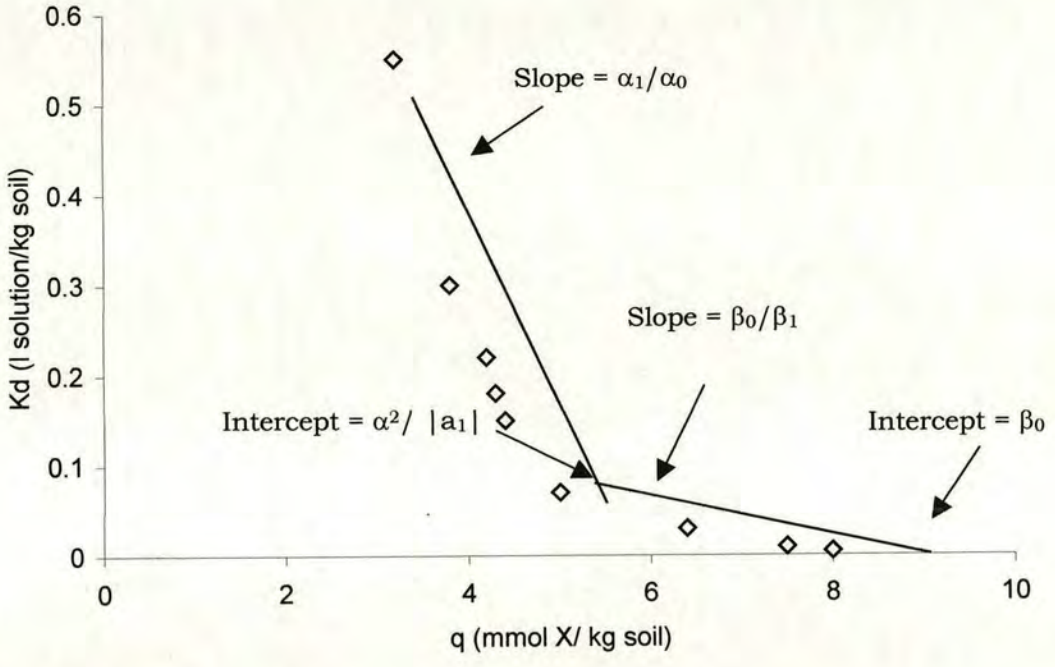

Fig. A.1.1 Illustration of the calculation of parameters for the two surface Langmuir equation according to the interpolation theorem (Sposito, 1982).

The graphs resulting from plotting c/q *versus* c (Figure A.1.2) suggested it was possible to use the 2 surface Langmuir equation to model Cr(VI) adsorption and parameters were calculated according to the interpolation theorem. The lines that best represented the L-shaped curved, according to the Pearson R^2 coefficient values were used (Figure A.1.3).

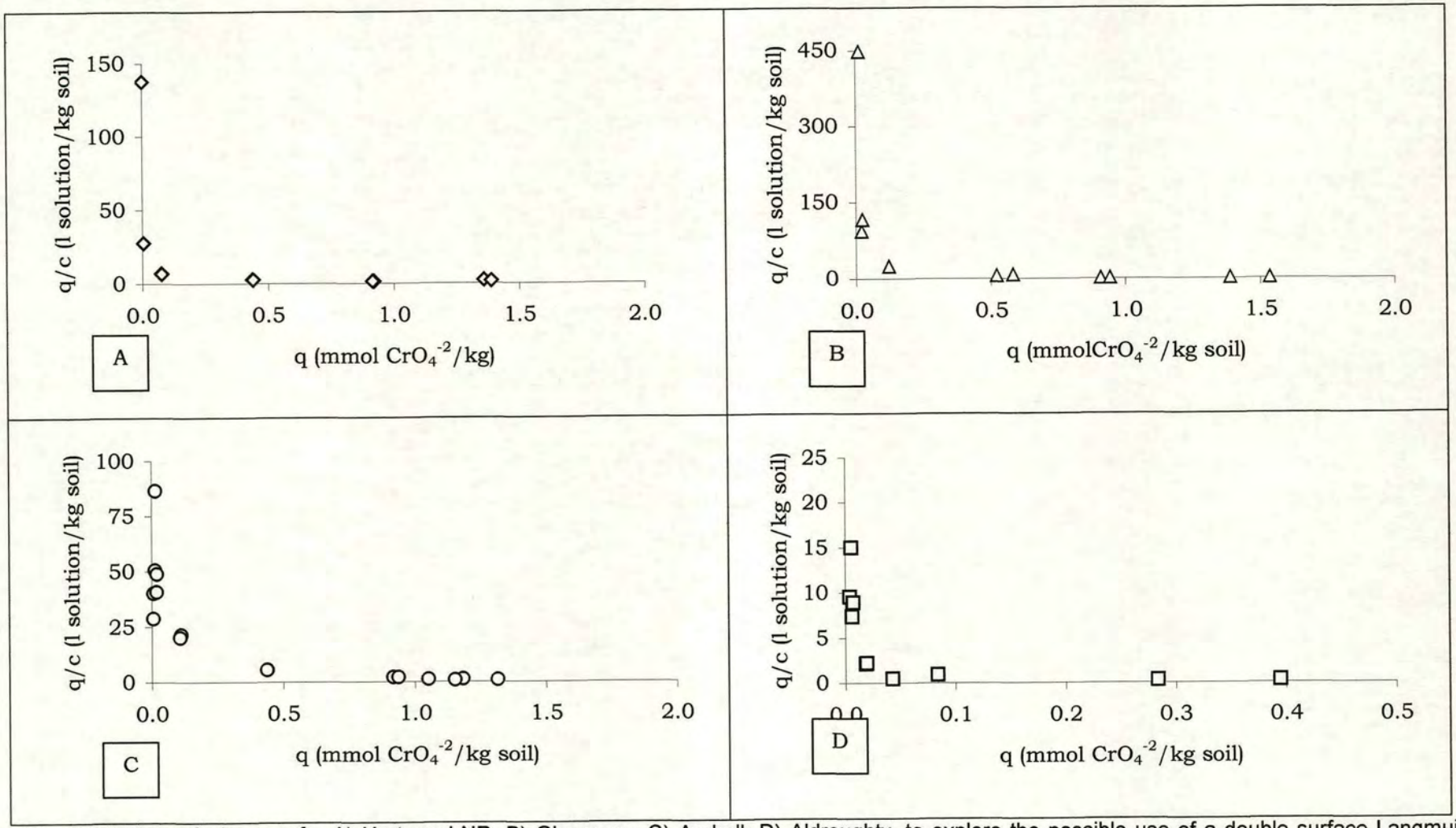

Fig. A.1.2 Plots of q/c *vs.* c for A) Hartwood-NR; B) Glencorse; C) Arnhall; D) Aldroughty, to explore the possible use of a double surface Langmuir equation to model chromate adsorption.

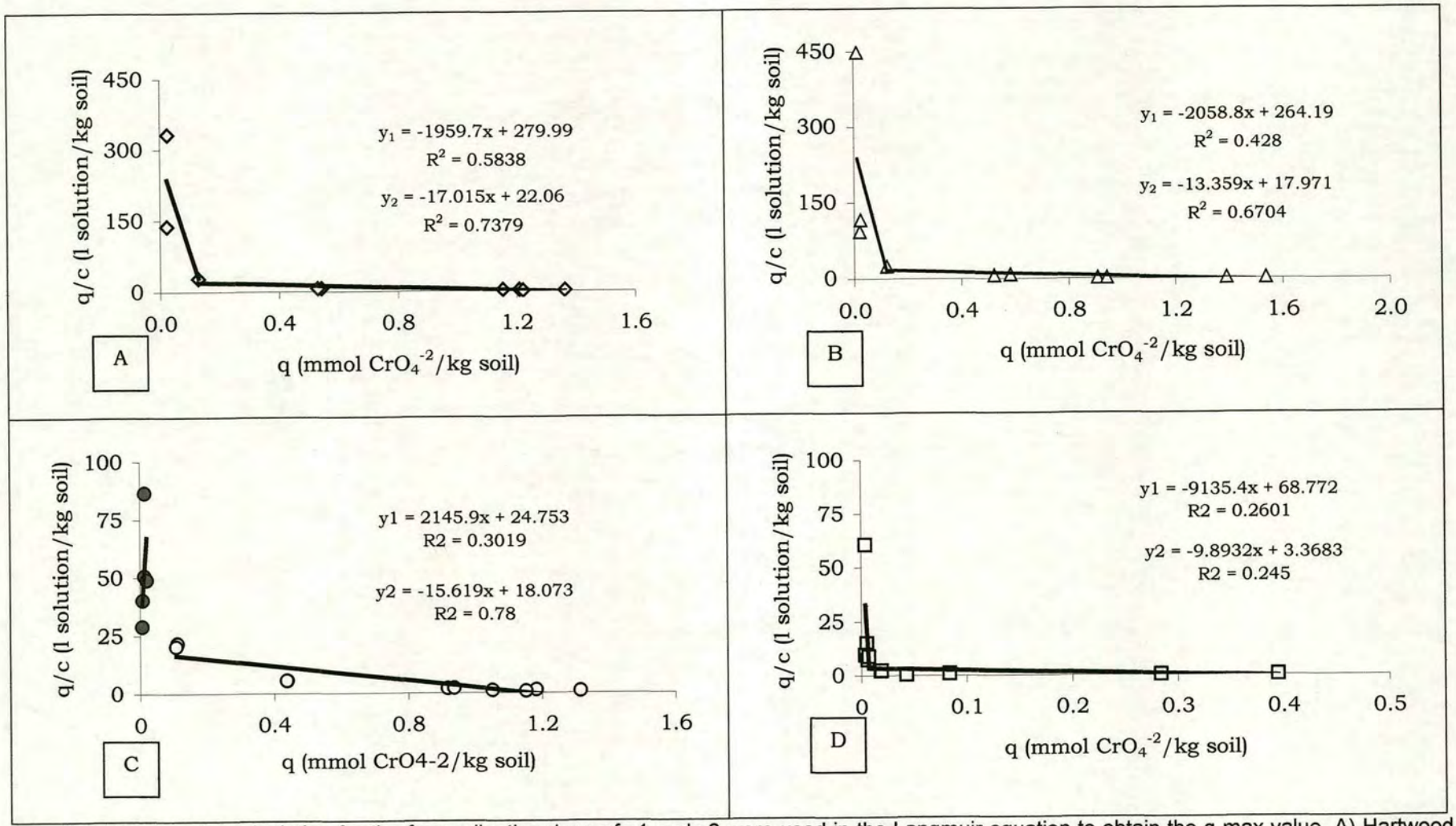

Fig. A.1.3 Parameter calculation for the four soils, the slope of y1 and y2 were used in the Langmuir equation to obtain the q max value. A) Hartwood, B) Glencorse, C) Arnhall, D) Aldroughty

Appendix 2

Calculations for Soil Water Holding Capacity for Microcosm Soils

Aldroughty in the fraction < 6 mm had initially 0.10 *g H_2O/g fresh soil* and 0.90 *g dried soil/g fresh soil*, therefore,

$$0.10 \frac{g\ H_2O}{g\ fresh\ soil} x \frac{1}{0.90} \frac{gfresh\ soil}{g\ dried\ soil} = 0.11 \frac{g\ H_2O}{g\ dried\ soil}$$

The amount of dried soil in the 50 g of fresh soil used to measure the WHC was:

$$50g\ fresh\ soil \times 0.90 \frac{g\ dried\ soil}{g\ fresh\ soil} = 45\ g\ dried\ soil$$

50 of fresh Aldroughty retained 13.5 g H_2O, equivalent to 0.3 gH_2O retained per g of dried soil. The water holding capacity of the soil was obtained by adding the water retained in the sample to the water already present in the sample (moisture content):

$$WHC = 0.3 \frac{g\ H_2O}{g\ dried\ soil} + 0.11 \frac{g\ H_2O}{g\ dried\ soil} = 0.41 \frac{g\ H_20}{g\ dried\ soil}$$

The amount of water present in the soil at 50% WHC would have been:

$$50\%\ WHC = 0.41 \frac{g\ H_20}{g\ dried\ soil} \times 0.5 = 0.205 \frac{g\ H_2O}{g\ dried\ soil}$$

Since the soil already contained 0.11 g H_2O/g dried soil, the difference, 0.095-g H2O/g dried soil, should be added. To take 400 g of Aldroughty to the 50% WHC,

$$400\ g\ fresh\ soil \times 0.90 \frac{g\ dried\ soil}{g\ fresh\ soil} \times 0.095 \frac{g\ H_2O}{g\ dried\ soil} = 34\ g\ H_2O,$$

i.e. 34 g H_2O had to be added to 400 g of fresh soil.

Calculations for the WHC of the three soils are summarised in Table A.2.1. Table A.2.2 shows data for the calculation of water needed to take 400g of each soil to 50% WHC. The total concentration of Cr (VI) to add to cover this range was calculated from:

$$tot\ CrO_4^{-2} = (q \times dried\ soil\ weight) + (c \times soil\ solution\ volume)$$

where q and c were obtained from the two-surface Langmuir equations for each soil (Table 4.2). Calculations for Aldroughty are shown below and data for Arnhall and Glencorse are included in Table 4.5.

In order to have 0.1mmol of CrO4-2 per L of soil solution and 0.16 mmol CrO-4 adsorbed per kg soil, the quantity of chromate that needs to be added to 0.4 kg of soil would be:

$$tot\ CrO_4^{-2} = (0.16 \frac{mmol CrO_4^{-2}}{kg\ soil} \times 0.36\ kg\ soil) + (0.1 \frac{mmol\ CrO_4^{-2}}{L} \times 0.074 L)$$

$$tot\ CrO_4^{-2} = 0.07\ mmol$$

where soil dried weight equals:

$$0.434\ kg\ sample \times 0.83 \frac{kg\ dried\ soil}{kg\ sample} = 0.36\ kg\ dried\ soil$$

0.434 kg being the original soil sample (0.4 kg) plus the water added (0.034 kg) to take the soil to 50% of its water holding capacity.
The volume H_2O at the same conditions was calculated by,

$$vol\ H_2O = (0.434 kg\ sample \times 0.17 \frac{kg\ H_2O}{kg\ sample}) \times \frac{1L\ H_2O}{1kg\ H_2O} = 0.074\ L$$

The total amount of chromate had to be added in the volume needed to take the soil to 50% of its WHC, therefore the stock solution of a given concentration was obtained from

$$0.07\ mmol\ CrO_4^{-2} \times \frac{1}{0.034\ L} = 1.91 \frac{mmol CrO_4^{-2}}{L}$$

Table A.2.1 Data for the calculation of WHC in the three soils used in the experiment

Soil	soil for WHC (g)	Dry soil/ g sample	gH_2O/ g sample	gH_2O/g dry soil	Soil dry weight (g)	g Water Retained in 50 g	g water retained by 1g dried s	WHC gH2O/gdried soil
AD	50	0.90	0.10	0.11	45.00	13.5	0.30	0.41
ARN	50	0.79	0.21	0.27	39.50	16.5	0.42	0.68
GC	50	0.79	0.21	0.27	39.50	15	0.38	0.65

Table 4.4 Data for the calculation of water needed to take 400 g of fresh soil to 50% of its WHC

Soil	current gH_2O/ g dried soil	target gH_2O /g dry soil	difference gH_2O/g dry sample	dry weight Soil (g)	gH_2O to add/remove	Final weight Sample (g)	dry matter Fresh soil (final)	gH_2O /g fresh soil (final)	gH_2O / g dry Soil
AD	0.11	0.21	0.09	360	34.0	434	0.83	0.17	0.21
ARN	0.27	0.34	0.08	316	24.0	424	0.75	0.25	0.34
GC	0.27	0.32	0.06	316	18.0	418	0.76	0.24	0.32

Table A.2.2 Stock solutions used to obtain given Cr(VI) in soil solution as calculated from adsorption models

Soil	Cr(VI)* [mg/L]	c [mmol/L]	q [mmol/kg]	Soil w [kg]	H_2O added [L]	Soil w final [kg]	H_2O [kg/kg]	Dry soil Final [kg]	Dry soil Final [Kg]	Soil S vol. [L]	CrO_4^{-2} [Mmol]	CrO_4^{-2} Stock mM
AD	5.2	0.1	0.16	0.4	0.034	0.434	0.17	0.830	0.360	0.074	0.07	1.91
AD	26	0.5	0.27	0.4	0.034	0.434	0.17	0.830	0.360	0.074	0.13	3.95
AD	52	1	0.30	0.4	0.034	0.434	0.17	0.830	0.360	0.074	0.18	5.35
AD	104	2	0.32	0.4	0.034	0.434	0.17	0.830	0.360	0.074	0.26	7.73
AD	156	3	0.32	0.4	0.034	0.434	0.17	0.830	0.360	0.074	0.34	9.90
AD	312	6	0.32	0.4	0.034	0.434	0.17	0.830	0.360	0.074	0.56	16.41
AD	520	10	0.32	0.4	0.034	0.434	0.17	0.830	0.360	0.074	0.85	25.09
AD	728	14	0.33	0.4	0.034	0.434	0.17	0.830	0.360	0.074	1.15	33.88
AD	1040	20	0.33	0.4	0.034	0.434	0.17	0.830	0.360	0.074	1.59	46.90
AD	1300	25	0.33	0.4	0.034	0.434	0.17	0.830	0.360	0.074	1.96	57.75
AD	1820	35	0.33	0.4	0.034	0.434	0.17	0.830	0.360	0.074	2.70	79.45
AD	2600	50	0.33	0.4	0.034	0.434	0.17	0.830	0.360	0.074	3.81	112.00
ARN	5.2	0.1	0.68	0.4	0.024	0.424	0.25	0.745	0.316	0.106	0.23	9.40
ARN	26	0.5	0.99	0.4	0.024	0.424	0.25	0.745	0.316	0.106	0.37	15.24
ARN	52	1	1.05	0.4	0.024	0.424	0.25	0.745	0.316	0.106	0.44	18.18
ARN	104	2	1.08	0.4	0.024	0.424	0.25	0.745	0.316	0.106	0.55	23.05
ARN	156	3	1.09	0.4	0.024	0.424	0.25	0.745	0.316	0.106	0.66	27.60
ARN	312	6	1.10	0.4	0.024	0.424	0.25	0.745	0.316	0.106	0.98	40.96

Soil	Cr(VI)* [mg/L]	c [mmol/L]	q [mmol/kg]	Soil w [kg]	H_2O added [L]	Soil w final [kg]	H_2O [kg/kg]	Dry soil Final [kg]	Dry soil Final [Kg]	Soil S vol. [L]	CrO_4^{-2} [Mmol]	CrO_4^{-2} Stock mM
ARN	520	10	1.11	0.4	0.024	0.424	0.25	0.745	0.316	0.106	1.41	58.72
ARN	780	15	1.11	0.4	0.024	0.424	0.25	0.745	0.316	0.106	1.94	80.84
ARN	1040	20	1.11	0.4	0.024	0.424	0.25	0.745	0.316	0.106	2.47	102.94
ARN	1300	25	1.11	0.4	0.024	0.424	0.25	0.745	0.316	0.106	3.00	125.03
ARN	1820	35	1.11	0.4	0.024	0.424	0.25	0.745	0.316	0.106	4.06	169.20
ARN	2600	50	1.11	0.4	0.024	0.424	0.25	0.745	0.316	0.106	5.65	235.45
GC	5.2	0.1	0.76	0.4	0.018	0.418	0.24	0.756	0.316	0.100	0.25	13.90
GC	26	0.5	1.13	0.4	0.018	0.418	0.24	0.756	0.316	0.100	0.41	22.62
GC	52	1	1.20	0.4	0.018	0.418	0.24	0.756	0.316	0.100	0.48	26.71
GC	104	2	1.25	0.4	0.018	0.418	0.24	0.756	0.316	0.100	0.60	33.09
GC	156	3	1.26	0.4	0.018	0.418	0.24	0.756	0.316	0.100	0.70	38.84
GC	312	6	1.28	0.4	0.018	0.418	0.24	0.756	0.316	0.100	1.01	55.91
GC	520	10	1.28	0.4	0.018	0.418	0.24	0.756	0.316	0.100	1.41	78.20
GC	624	12	1.29	0.4	0.018	0.418	0.24	0.756	0.316	0.100	1.61	89.48
GC	1040	20	1.29	0.4	0.018	0.418	0.24	0.756	0.316	0.100	2.41	134.09
GC	1300	25	1.29	0.4	0.018	0.418	0.24	0.756	0.316	0.100	2.92	161.98
GC	1820	35	1.29	0.4	0.018	0.418	0.24	0.756	0.316	0.100	3.92	217.71
GC	2600	50	1.29	0.4	0.018	0.418	0.24	0.756	0.316	0.100	5.42	301.31

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